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THE EFFECT OF STAPHYLOCOCCUS AUREUS TOXIN ON THE KIDNEY

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PLATE 1

(Received for publication, October 5, 1934)

The first report of the production of a toxin by the *Staphylococcus aureus* that damages the kidney was given by Neisser and Levaditi (1) in 1900. These authors described the kidneys of rabbits that had received injections of the toxin as being greyish white with irregular red areas. The external portion of the cortex was white, opaque and semipurulent. The histologic study revealed two inflammatory zones with fragmenting polymorphonuclear leucocytes surrounding a third intermediate zone of necrosis, within which the cells of the convoluted tubules were necrotic, their nuclei pale staining and sometimes fragmented. The glomeruli were engorged, filling the capsule of Bowman; some glomeruli were necrotic and surrounded by an albuminous or hemorrhagic exudate. The epithelium of Bowman's capsule was in some instances destroyed, in others, proliferated. The most striking lesion was in the small blood vessels below or at the border of the inner zone. These vessels were obstructed by masses formed of fragmented leucocytes, or by fibrin thrombi. Later, Neisser and Wechsberg (2), and more recently, Forssman (3), and Rigdon, Joyner and Ricketts (4) have reported essentially the same results following the injection of toxin into rabbits.

This paper deals with the effect of the staphylococcus toxin on the kidneys of rabbits and cats. Much of the material described here was collected by Weld and Gunther (5) and was briefly alluded to by them in their report in 1931.

Methods

Toxins were prepared according to the method described by Parker, Hopkins and Gunther (6). The organisms from which the toxins were made were obtained from different sources. The strength of toxin produced varied. The toxin was tested for its potency and the suitable dose determined that would produce renal lesions. In every instance it was injected intravenously.

The tissues were fixed in Zenker's solution without the addition of either acetic acid or formalin. The sections were stained in a variety of ways: hematoxylin-

eosin, Weigert's elastic tissue stain followed by hematoxylin-eosin, Mallory's phosphotungstic acid hematoxylin, Gram's stain and McGregor's azan carmine.

Effect on Rabbits

Eighteen young rabbits were used in the experiment. A dose of the toxin sufficient to produce lesions in the kidneys was given and the animals were killed at varying intervals. Thus, it was hoped to follow the development of the lesions.

One rabbit was given 0.3 cc. of the toxin intravenously; two others 0.5 cc. each. They were killed at the end of 1 hour. No definite alterations could be made out in the kidneys. The time interval was apparently too short.

Two animals were killed at the end of 2 hours after the toxin injection. One had received 0.15 cc., the other 0.18 cc. of toxin. Areas of injection of the cortex of the kidneys were found in the gross. Histologically, the capillaries in many of the glomeruli were dilated. The epithelial cells of the convoluted tubules were swollen, pale staining and the cytoplasm finely granular. Fibrin could not be found in the blood vessels.

Three animals were killed 4 hours after receiving the toxin, in doses of 0.15, 0.2 and 0.3 cc., respectively. On gross examination the kidneys were found to be swollen and tiny hemorrhages could be seen in the cortex just beneath the capsule. The histologic examination revealed definite changes. The tubules in the superficial portion of the cortex were spread apart by hemorrhages. In each of these animals the capillaries of many of the glomeruli were dilated. In the animal that received 0.15 cc. of the toxin, in addition to dilatation of the glomerular capillaries, there were numerous chromatin particles from the fragmented nuclei of the glomerular endothelial cells. The capsular spaces were reduced in size by the dilatation of the capillaries, yet most of the capsular spaces, whether the capillaries were dilated or not, contained granular precipitate. The arterioles leading to the engorged glomeruli were distended with blood and about these vessels hemorrhages were sometimes seen. In the two animals that received the larger doses, some of the arteries extending upward through the cortex that gave off the afferent glomerular arterioles, were found to be greatly dilated, their walls were necrotic and infiltrated with blood. Their lumina contained some fibrin. The epithelial cells of the convoluted tubules were swollen, pale and finely granular. Granular precipitate was present in the lumina of the tubules. In one animal with the severe arterial damage, some loops of Henle were found lined by necrotic epithelial cells. There was no inflammatory reaction present in these kidneys.

One animal was killed 8 hours after receiving 0.15 cc. of toxin. The kidneys were mottled with small hemorrhages. The histologic changes were essentially the same as in the preceding group except that the damage was greater. Many arteries in the cortex were necrotic and fibrin was present in their lumina.

Six rabbits were each given a single dose of toxin varying from 0.1 to 0.25 cc. One animal was killed after an interval of 12 hours, one at the end of 20 hours, one at 22 hours and three at 24 hours. The kidneys of each animal were severely in-

jured. Hemorrhages were present in the cortex, and in some kidneys small opaque areas with a faint yellow tinge were found.

In the animal killed at the end of 12 hours, the histologic study of the kidneys revealed hemorrhages between the tubules, engorged glomeruli, some of which had fragmenting nuclei, and hemorrhage into the capsular space. Fibrin was present in some of the capillary loops and in a few capsular spaces. Many arterioles had necrotic walls infiltrated with red blood cells and fibrin, and compact fibrin was adherent to their inner surfaces. The epithelial cells of many convoluted tubules were so swollen that they obliterated the lumen of the tubules; their nuclei were shrunken or had disappeared. Many of these cells were obviously necrotic. Leucocytes had collected about these tubules. In the lumina of other convoluted tubules, fibrin was found and the epithelium was necrotic.

The kidneys of the animal sacrificed at the end of 20 hours showed essentially the same changes as those found in the animal killed 12 hours after injection.

In the animal killed at the end of 22 hours, a wide band of necrosis in the cortex was found, bordered by interstitial hemorrhage. At the outer margin of the necrotic area, the epithelial cells of the tubules were swollen and their nuclei had disappeared. The capillaries of the glomeruli were greatly distended; in many, the endothelial cells had disappeared and the epithelium over them was swollen. In the central part of the necrotic area, the epithelial cells of the tubules stained palely; their nuclei were fragmenting. These tubules were somewhat separated from each other by edema fluid. There was little hemorrhage between them. The glomerular capillaries were not so distended as in the outer zone; they contained laked blood and the nuclei of the endothelial cells were pyknotic. Similar changes were found in the epithelial cells of Bowman's capsule. At the junction of the outer and inner zones of the necrotic area fragmenting leucocytes were often seen densely packed between the tubules. Many of the arteries in the central part of the necrotic area were necrotic and their lumina were filled with fibrin while red blood cells had escaped into their walls.

Two of the animals killed at the end of 24 hours presented renal changes identical with those described in the animal sacrificed at 22 hours.

In the other animal killed after 24 hours, the renal damage was not so extensive. There were sharply localized cortical areas in which some of the glomeruli showed dilated capillaries with loss of endothelium and the epithelium over these loops was swollen. The cells of the convoluted tubules were necrotic, swollen and anuclear. In a few of these areas leucocytes had begun to accumulate. No lesions could be found in the arteries.

Two rabbits were given 0.4 and 0.5 cc. of toxin, respectively, and died at the end of 2 days. The changes were identical with those described in the animals killed at the end of 22 hours except that more leucocytes had collected at the margin of the necrotic zone.

One animal, given 0.2 cc. of toxin, was allowed to live for 3 days. The changes in the kidneys were not so advanced. The arteries were normal. Many glomer-

ular capillaries were distended; in a few of them the nuclei of the endothelial cells were fragmenting. An occasional glomerulus was found with only a single loop distended to a size greater than that of an uninvolved glomerulus, and crowding the other loops of the glomerulus against Bowman's capsule. Other glomeruli were bloodless and shrunken. The epithelium over the involved glomeruli was unusually prominent. The epithelium of the convoluted tubules about the injured glomeruli had undergone necrosis; fibrin was present in the lumina of these tubules. Some of them were being relined with flattened epithelium and necrotic epithelial cells filled the lumen, often in a compact mass.

Effect on Cats

It was deemed advisable to study the effect of the toxin in an animal other than the rabbit; conceivably the changes produced might be different. Ten young cats weighing from 460 to 1550 gm. were selected. The toxin was injected into one of the leg veins, usually the femoral vein. The amount of toxin injected varied from 0.12 cc. to 1.5 cc. Most of the animals lost weight. None was killed. In Table I is shown the weight of the animal, the amount of toxin given and the length of time of survival.

Gross Changes in the Kidneys.—When small doses were given, the kidneys presented no gross abnormality. Thus, in seven of the animals the kidneys appeared normal. In one of the other three animals the cortical vessels were greatly engorged; in the other two petechiae were present in the cortex. The absence of striking gross lesions was in contrast to the abnormal appearance of the kidneys of the rabbits that had been given the toxin.

Histologic Alterations in the Kidney.—The main effect of the toxin was upon the glomeruli and especially upon those glomeruli in the inner half of the cortex. Usually the capillary loops were not uniformly involved in any single damaged glomerulus. The extent of the damage varied according to the amount of the toxin given.

The earliest detectable change was the dilatation of some of the capillary loops of the glomerulus, often to three times the normal calibre. The red blood cells in these distended loops were frequently pale and in some instances, delicate strands of fibrin were present in the capillary lumen. The endothelial cells lining these distended capillaries were pale staining, their outlines vague and their nuclei fragmented. Occasionally a mitotic figure could be found in the endothelium. The epithelial layer over these damaged capillary loops could not be made out. The capsular space was empty. No alterations could be detected in the epithelium of the tubules. The arterioles were normal.

TABLE I

Cat No.	Weight	Amount of toxin	Interval between last injection and death	Renal lesions
	gms.	cc. 1932		
AB2	950	0.25 June 2 0.25 June 6 0.25 June 10	4 days	Slight
AB6	720	0.12 Oct. 21 0.25 Oct. 24 0.25 Oct. 26	Less than 24 hrs.	Slight
AB5	925	0.25 June 17 0.25 June 20 0.25 June 23 0.25 June 27 0.5 July 1 0.5 July 8	Less than 24 hrs.	Moderate
AB9	880	1933 0.15 Feb. 24 0.25 Feb. 26 0.4 Mar. 1 0.6 Mar. 6 0.8 Mar. 9	4 hrs.	Moderate
AB12	570	0.125 Mar. 9 0.3 Mar. 14	22 hrs.	Moderate
AB1	700	1932 0.5 May 27 0.5 May 31	4 hrs.	Severe
SC5	1350	1.5 Apr. 6 2.5 Apr. 11	Less than 24 hrs.	Severe
AB3	1050	0.25 June 17 0.5 June 20 0.5 June 23	5 hrs.	Severe
AB4	950	0.25 June 17 0.25 June 20 0.25 June 23	Less than 24 hrs.	Severe
AB8	460	1933 0.07 Mar. 3 0.25 Mar. 6	Less than 24 hrs.	Severe

When a somewhat larger amount of the toxin was given the distention of the glomerular capillaries was more marked though frequently only two or three loops would be involved. These loops were so dilated that at times the diameter was greater than that of an entire uninvolved glomerulus. A few endothelial cells could be found in some of the injured glomerular capillaries, and some of them had no epithelial covering. Fibrin was present in some of these capillaries. The capsular space was partially or completely obliterated by the dilated glomerular capillaries. About many glomeruli the capsular space was filled with a solid coagulum that in part was composed of fibrin. The coagulum extended down into the first part of some of the proximal convoluted tubules. The epithelial cells were absent where the distended glomerular capillaries pressed against the capsule of Bowman. In other places the capsular epithelial cells were often larger than normal and stained deeply with hematoxylin, as though recently regenerated. When the coagulum filled the space, the capsular epithelium had disappeared for the most part. There was some flattening of the epithelium by the coagulum present in the first part of some of the proximal convoluted tubules. Other than this the only alteration in the epithelium of these tubules that could be detected was fine eosin-staining granules in the cytoplasm. The arterioles were normal.

When the damage was most severe, a compact layer of fibrin was pressed against the inner surface of the basement membrane of the distended capillaries of the glomeruli and delicate fibrin strands often extended toward the center of the lumen (Fig. 1). The endothelium had entirely disappeared in these loops, or cells resembling endothelium were occasionally seen extending along the fibrin. The red blood cells in the lumina of the distended capillaries stained normally though in some instances they were fused together. Sometimes only a single capillary loop was involved, the others being crowded aside, compressed against the capsule of Bowman and bloodless. The dilated glomerular capillary in some instances was covered by proliferating epithelium; over others the epithelium was lacking. The capsular epithelium was found sometimes to have proliferated, and mitotic figures were occasionally present in these cells. Rarely, a moderate number of polymorphonuclear leucocytes was collected at the periphery of the dilated capillary loop. The capsular space was reduced or almost obliterated by the dilatation of the capillaries; frequently what remained of it was filled with a compact mass of eosin-staining material, in part fibrin, that extended into and filled the lumen of the proximal convoluted tubule. Sometimes red blood cells could be distinguished in this coagulum. The tubules immediately adjacent to many involved glomeruli were compressed, seemingly because of the distention of the capsule of Bowman by the dilated glomerular loops. The epithelium of some of the convoluted tubules showed definite necrosis.

In one animal the epithelium of several large groups of tubules in the pyramid was necrotic. Hemorrhages were present in the interstitium of the cortex in some of the kidneys. An acute inflammatory reaction was rarely observed in the cortical interstitial tissue of the kidneys.

Only one animal in which the renal damage was severe showed any change in

the arterioles. In this instance some of the afferent arterioles just outside the injured glomeruli contained compact fibrin similar to that in the altered capillary loops.

The basement membranes of the glomerular capillaries were always intact though stretched and thinned out in the distended loops. In none of the animals could any other defect in this membrane be discovered.

DISCUSSION

It is obvious from the experiments reported here and those of previous observers that a toxin is elaborated by the hemolytic *Staphylococcus aureus in vitro* that produces severe damage in the kidneys of rabbits and cats. There is, however, some disagreement as to the pathogenesis of the lesion. Levaditi and Neisser, and Neisser and Wechsberg believed the lesions to be in the nature of infarcts. Rigdon, Joyner and Ricketts were of the opinion that the toxin injured the epithelial and endothelial cells of the glomeruli and the epithelium of the convoluted tubules and loops of Henle. They also stated that the endothelial cells of the glomeruli appeared more resistant to the toxin than the epithelium of the tubules.

In our own material from rabbits definite lesions could be found in glomeruli as soon as 4 hours after the injection of the toxin and at this time some arteries, also, were necrotic. The changes in the vascular elements of the kidneys were further advanced than those in the epithelium of the tubules. When the arteries were occluded by fibrin the circulation must have ceased through certain portions of the cortex and it would seem most plausible that this occlusion determined in many of the kidneys the widespread cortical necroses that are indeed of the nature of infarcts. It is of interest that in the rabbits in which the arteries were not involved, the widespread infarct-like lesions were absent. As regards the changes in the epithelium of the tubules, these could well be accounted for by the interference of the glomerular circulation resulting from the damage to the capillary loops.

In the kidneys of the cats the arterioles were seldom injured, the initial damage being in the glomerular capillaries. It is difficult, however, to explain the varying degree of this damage in the different loops of a glomerulus. The changes in the epithelium of the tubules were never so intense as in the glomerulus; the most reasonable explanation for this is that the tubules were involved secondarily. The

fact that the arteries were not injured probably accounts for the absence of the infarct-like lesions, so commonly found in the rabbit's kidneys.

No lesions comparable to those in the kidneys of the rabbits and cats could be found in the other viscera of these animals. The toxin when injected intravenously appears to damage the kidney selectively and, furthermore, to damage primarily the blood vessels of the kidney.

CONCLUSIONS

1. The hemolytic *Staphylococcus aureus* elaborates a toxin *in vitro* that when injected intravenously produces lesions in the kidneys of rabbits and cats.

2. The toxin injures primarily the blood vessels of the kidney.

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EXPLANATION OF PLATE 1

FIG. 1. Cat AB1. Given two doses of toxin, 0.5 cc. each, at interval of 4 days.

(a) Two uninvolved glomeruli. Stained with hematoxylin and eosin. $\times 360$.

(b) Involved glomerulus. One capillary loop is greatly distended; it contains much fibrin. Other glomerular capillaries are compressed against capsule of Bowman. Capsular space filled with compact coagulum that is composed in part of fibrin. The coagulum extends down into the first portion of the convoluted tubule. Stained with hematoxylin and eosin. $\times 360$.

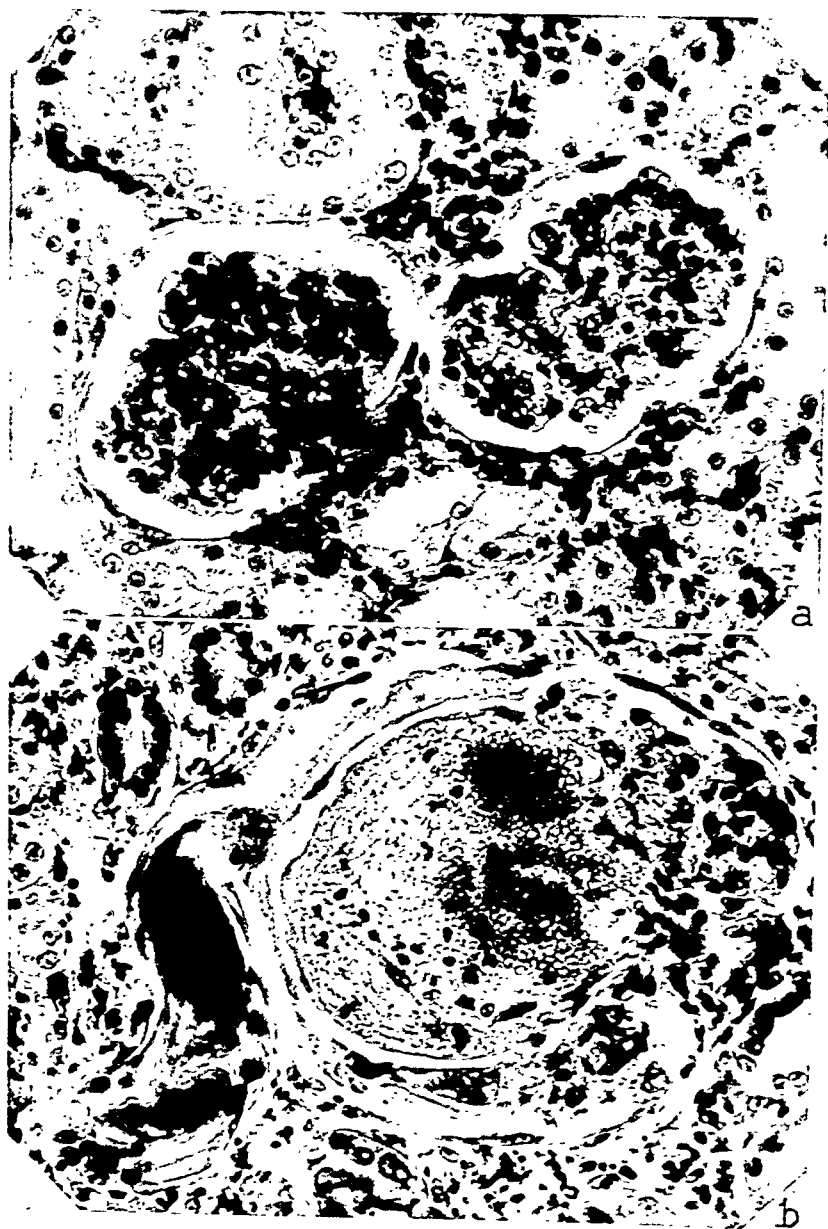


FIG. 1

(VonGlahn and Weld: Effect of *S. aureus* toxin on the kidney)

CORTIN PROTECTION AGAINST ANAPHYLACTIC SHOCK IN GUINEA PIGS

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(Received for publication, October 13, 1934)

The adrenal glands have long been suggested as having a protective action in anaphylactic shock. Anderson and Schultz (1) and Pelz and Jackson (2) showed the importance of adrenalin in early stages of experimental shock, and extensive clinical experience seems to warrant its continued use as an emergency treatment. Kepinow (3), working with guinea pigs, and Flashman (4), working with rats, both found that a smaller dose of antigen induced fatal shock in adrenalectomized animals. The latter reported that adrenalectomy either before or after sensitization had the same effect, which was proportional to the degree of adrenal insufficiency present. Wyman (5) confirmed these results, and his work led him to believe that the lack of medullary tissue was the deciding factor in the increase of susceptibility. Perla and Morston-Gottesman (6), working with the closely related histamine shock, found that the resistance of adrenalectomized rats to histamine was raised by the administration of a number of doses of cortin.

We were led to determine what protective effect adrenal cortex extracts might have in experimental anaphylactic shock in non-operated animals. On theoretical grounds our attempt was feasible. In corticoadrenal insufficiency there is a disturbed electrolyte metabolism which accompanies changes in cell permeability. This is corrected by parenteral administration of cortin (7). In anaphylactic shock also, the syndrome can be explained, at least partially, on a disturbed permeability basis. Furthermore, in both experimental adrenal insufficiency (8) and anaphylactic shock (9-13) many of the symptoms are alleviated or postponed by the administration of sodium salts.

Material and Methods

Fifty-eight young guinea pigs weighing between 250 and 400 gm. were used. They were kept in large cages and fed a diet of oats, hay, lettuce, and water. Nine were used for variable dosage tests, thirty-three were cortin-treated, and sixteen were test controls.

Purified, three times recrystallized egg albumin,¹ was chosen as a sensitizing and shocking agent by reason of its being a single antigen. A sensitizing dose of 1 mg. of egg albumin was given intraperitoneally to all animals. The interval between sensitizing dose and shocking dose was in each case 14 days.

A few preliminary experiments using from 5 to 40 mg. doses showed that 13 mg. given intraperitoneally would cause fatal anaphylactic shock in less than 35 minutes. This was the amount used for all controls and all cortin-treated animals. Controls were included whenever a series of cortin-treated animals received shocking doses.

The cortin was prepared by one of us from fresh beef adrenals and was made up in normal saline solution. Extract prepared according to two different methods did not alter the results significantly. Both types had been proven potent by administration to adrenalectomized cats. Each guinea pig received a single dose of 3 cc. of a solution of the hormone in the muscles of a hind leg. The interval between injection of cortin and the injection of the shocking dose varied in different cases between 1 hour and 27 hours. Some of the controls received Ringer solution in amounts equivalent to the cortin given, with no differences in their reactions.

RESULTS

Sixteen control animals were used and fourteen of these died in less than 35 minutes (Table I). A typical case (B 15) showed the usual first signs of shock and was weak and recumbent within 5 minutes after the intraperitoneal injection of the antigen dose. The characteristic spasmodic breathing began 5 minutes later, increased in intensity, and 24 minutes after the administration of the albumin the animal was dead. Two animals that survived (B 41 and B 42) were found at autopsy to have adrenals larger than normal. Two others intended for use in the control series survived longer than $\frac{1}{2}$ hour. The autopsies showed definite lung infections. All other animals that had been kept with these were autopsied carefully after use, and

¹ This was kindly furnished by Dr. M. Heidelberger of the Department of Biological Chemistry, whom we take this opportunity to thank. We also wish to thank Dr. Beatrice Seegal of the Department of Bacteriology for advice at the beginning of these experiments, and Mr. John S. Wolf for his help in some of the experimental procedures.

the results from control and cortin-treated guinea pigs that showed any lung consolidation at all were discarded.²

The time interval between the administration of cortin and of egg albumin proved to be important (Table I). The greatest amount of protection was seen when the cortin was given from 2 to 6 hours before the albumin. If the interval was shorter or if it was much longer, the degree of protection was greatly diminished. There was some evidence of protection when the period was only a few hours longer, as in the 11 hour group.

Of the sixteen animals given cortin 2 to 6 hours before the antigen, nine survived indefinitely, two lived about an hour, and the other five died in less than 35 minutes.

In a 1 to 1½ hour group of six animals, the longest survival period was ¾ hour. In the 11 hour group of four guinea pigs, one survived indefinitely, one lived 1 hour, and two lived less than 35 minutes. A 17 to 27 hour group of seven showed three survivals for approximately an hour.

In the seven guinea pigs surviving 45 to 64 minutes the appearance of the behavior usually considered as typical of anaphylactic shock was delayed in each case until a few minutes before death. Up to that time they were quite weak and lay quietly on their sides, but were strong enough to move about fairly actively when repeatedly disturbed.

The ten animals that survived did not pass into marked shock. One showed no weakness, and no signs of shock whatsoever. The others were weak for a period which was at the longest 15 minutes, and then began to show slight signs of shock. These persisted in some cases for a considerable time; in others they were evanescent and consisted only of one or two gasps or snuffles. Usually weakness continued for a few minutes after the signs of shock had disappeared. By 1½ hours after the injection of the albumin all the animals had recovered sufficiently to be interested in eating and drinking.

² The infected animals when considered by themselves confirm the results of the non-infected groups, but were not included in our tables nor in the total of animals used. The results obtained seem to confirm the view based on both experimental and clinical evidence that allergic manifestations are inhibited by general infection.

TABLE I

Cortin Protection against Anaphylactic Shock

1 mg. sensitizing dose and 13 mg. shocking dose given intraperitoneally in all cases. All treated animals received a single intramuscular injection of 3 cc. of cortin.

Control animal	Survival time	Cortin-treated animal	Interval between cortin and shocking injections	Survival time
	<i>min.</i>		<i>hrs.</i>	<i>min.</i>
B 33	18	B 34	1	31
B 32	26	B 31	1	33
B 35	26	B 36	1	38
B 44	25	B 43	1½	24
		B 48	1½	27
		B 46	1½	46
B 13	23	A 21	2½	Survived
B 15	24	B 20	3½	18
B 12	26	B 16	3½	27
B 14	28	B 17	3½	34
		B 19	3½	Survived
		B 18	3½	Survived
		B 11	3½	Survived
B 3	13	B 1	3½	64
B 7	34	B 2	3½	Survived
		B 8	3½	Survived
A 36	17	A 33	4½	Survived
		A 32	4½	Survived
A 3	5	A 7	3½	56
A 2	10	A 5	4½	Survived
		A 4	5½	20
		A 1	5½	23
{ B 33	18	B 38	11	17
{ B 32	26	B 37	11	30
{ B 35	26	B 40	11	60
		B 39	11	Survived
B 50	20	B 49	17	25
B 41	Survived	B 47	17	31
B 42	Survived	B 45	17	55
{ B 3	13	B 6	24	14
{ B 7	34	B 10	24	60
		B 9	24	63
		A 6	27	41

* Same controls as above.

Eight of the cortin-treated animals that survived a usually fatal antigen dose were injected again with 13 mg. of egg albumin after 14 days (Table II). Seven of these died in shock; the other became weak quickly, showed first signs of shock at 5 minutes and had difficulty in breathing. It was killed after $1\frac{3}{4}$ hours, at which time it was very weak and showed marked dyspnea. This experiment shows that the cortin protection is not a permanent desensitization and also indicates that the original survival was a result of treatment and not the chance individual peculiarity of the animals.

TABLE II
Fate of Ten Cortin Survivors

Animal No.	Survival time
	min.
A 5	10
B 39	28
B 2	30
B 11	32
B 19	37
A 21	68
B 8	83
B 18	Signs of shock in a few minutes. Killed after $1\frac{3}{4}$ hrs. Autopsied (negative) Autopsied (negative)
A 32	
A 33	

Seven of eight given second injection died of shock. This shows that cortin protection is not prolonged and also that these animals were sensitive to shock.

Studies have been made on the microscopic anatomy of the adrenal glands from control, untreated guinea pigs dying of shock. These suggest that there is an acute demand (14) on the adrenal cortex during shock. A few preliminary blood chemical determinations also indicate that changes are taking place which can to some extent be remedied by the survival hormone of the adrenal cortex. We are continuing these studies on anaphylactic shock and on the relation of the adrenal cortex to other allergic phenomena.

DISCUSSION

The combined evidence of many investigations shows that the adrenal glands are involved in natural anti-anaphylactic efforts of the

body. We feel, however, that emphasis laid on the rôle of the medulla has obscured the part played by the cortex. The foregoing experiments show that corticoadrenal extracts by themselves have a definite anti-anaphylactic value. Cortin has also been used with some success in the closely related histamine shock (6) and in a few cases of traumatic shock (15).

We believe that the natural protective action involves first the cortex and then the medulla. The speed, duration and extent of the changes produced by adrenalin and cortin have been shown to differ in respect to carbon dioxide capacity (7) and carbohydrate mobilization (16). The same thing appears to be true in anaphylaxis. The use of adrenalin has been found to be most effective at the time of shocking, whereas in our experience cortin is best used several hours earlier. Cortin appears to have a general effect on the syndrome of shock, whereas adrenalin (as well as atropine) seems to have a symptomatic effect on allergic phenomena.

In experimental anaphylactic and other types of shock, certain blood changes have been described. There is a lowered carbon dioxide capacity (10, 17), a decreased plasma volume (18, 15), and a rise in serum potassium (19). In experimental adrenal insufficiency also, one finds a low carbon dioxide capacity (7), diminished plasma volume (18, 15), and a high serum potassium (20, 7). Conversely, cortin injection is followed by increased carbon dioxide capacity (21, 22, 7) and plasma volume (15) and a lowered serum potassium (7). Furthermore, the low carbon dioxide capacity in adrenal insufficiency is associated with a loss of serum sodium and the latter is also increased after cortin administration (7). In experiments on both anaphylactic shock (9-13) and adrenal insufficiency (8) it has been found that the use of relatively large amounts of sodium salts was of some benefit. There seems to be a similarity in the blood changes of both conditions.

In the absence of more definite information, we suggest that cortin protects from anaphylactic shock by reason of its regulatory effect upon ionic and other changes dangerous to the health of the organism.

SUMMARY

Studies have been made on a possible protective action of cortin in the prevention of anaphylactic shock induced by crystallized egg albumin.

Of sixteen control guinea pigs only two survived.

Sixteen of thirty-three cortin-treated animals received hormone at the optimum period of from 2 to 6 hours before the shocking dose. Nine survived and two others showed some protection. In groups treated at other time intervals the evidence of protection is inconclusive.

2 weeks after the first test the cortin-treated survivors succumbed to a second antigen dose, which was not preceded by cortical extract.

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ON THE PRESERVATION OF TYPHUS FEVER RICKETTSIAE IN CULTURES

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INTRODUCTION

In 1930 Nigg and Landsteiner (1) reported the cultivation through successive generations of the rickettsiae of typhus fever in tissue cultures as well as in a medium essentially like that of Maitland and Maitland (2) for vaccine virus. The cultures were virulent and contained large numbers of rickettsiae. A full account of the method and results was presented in a later paper (3). In 1931 Sato (4) reported the cultivation of the virus of typhus fever through thirteen generations using Descemet's membrane imbedded in coagulated plasma, but he was never able to demonstrate rickettsiae morphologically in such cultures with certainty. Subsequently, Pinkerton and Hass (5, 6) described the cultivation of typhus fever rickettsiae by imbedding infected tissue in coagulated plasma. They state that in the majority of cases the rickettsiae whilst numerous in the first generation cultures disappeared quite rapidly in successive transfers. They were able to maintain such cultures through successive transfers for 52 days, using an incubation temperature of 32°, stating that such cultures kept at 37.5°C. for 11 days were non-virulent and showed no rickettsiae histologically. In 1932 Hoshizaki (7) reported on the cultivation of rickettsiae from Manchurian typhus fever, using infected tunica imbedded in coagulated plasma on a cover-glass. He was unable to carry such cultures beyond the fifth generation. During the same year Yu (8) was able to cultivate typhus fever rickettsiae also using infected tissues imbedded in coagulated plasma. Although he carried such cultures through six generations, he found rickettsia-like organisms in only one culture. In 1933 Kemp (9) reported briefly that he had duplicated the work of Nigg and Landsteiner on the cultivation of typhus fever rickettsiae.

Since the first papers from this laboratory on the cultivation of typhus fever rickettsiae, various phases of this study have been continued. The present study is concerned with the preservation of the viability and virulence of the typhus fever rickettsiae in cultures and infected tissues.

Several strains of typhus fever rickettsiae, isolated in September,

1931, from guinea pigs infected with the murine type of typhus fever,¹ have been carried without interruption in cultures by successive transfers, using the method described in an earlier paper (3). During the 3 years which have elapsed since these cultures were initiated, there has been no evidence of diminution in virulence, the febrile reactions and scrotal lesions obtained in guinea pigs with the last generations being indistinguishable from those produced by the passage strain which has been maintained in animals. Rickettsiae are, as a rule, found in enormous numbers in the tissue bits in routine cultures (the stained preparations resembling those of ordinary bacterial cultures in this respect) and are readily demonstrable in the tunica exudate in guinea pigs infected with these cultures.

EXPERIMENTAL

A murine strain of typhus fever was used throughout this work. For the preparation and staining of the cultures, reference is made to the previous paper (3). In these experiments the serum-Tyrode medium was used as described except that the ratio of serum to Tyrode solution was 1 to 5 respectively and phenol red (0.0012 per cent) as an indicator was added to the latter. In the later generations the stock cultures were transferred routinely about once a month.

All of the flasks of each experiment summarized in Tables I and II were prepared simultaneously, using the same inoculum and medium constituents, and placed in the same incubator for 10 to 14 days, at the end of which time slides were made from 2 or 3 flasks to determine whether satisfactory multiplication had occurred. The remaining unopened flasks were then kept at the following temperatures: 37°, 20°, -4° and -20°C. After the intervals given in the protocols, the cultures were transferred to fresh medium and inoculated intraperitoneally into male guinea pigs to determine both the viability and virulence of the cultures. The febrile reaction and the development of scrotal lesions in each guinea pig were charted. When the symptoms had subsided, the animal was killed, and an emulsion of the brain was used to transfer the infection to other male guinea pigs. At the first indication of infection in these latter animals the scrotal lesions were examined for rickettsiae, and blood cultures were made from the heart's blood to rule out concomitant infections. Microscopical examination for rickettsiae and blood cultures were not made on the first animals injected with culture material as that would have necessitated killing the animals in the early stage of the disease, so that the course of the febrile and scrotal reactions would have been incomplete.

¹ Wilmington strain for which we are greatly indebted to Dr. W. G. McCoy, Director of the United States Public Health Service.

RESULTS

In Table I are given the results of comparative tests at -4°C. , 20°C. and 37°C. All the cultures in this experiment were prepared at the same time and incubated at 37°C. for 10 days after which they were kept at the various temperatures and tested after the intervals indi-

TABLE I
Survival of Typhus Fever Rickettsiae in Cultures (60th Generation) Stored at -4°C. , 20°C. and 37°C.

Temperature °C.	10 days				4 wks.			12 wks.			
	Culture	Guinea pig			Culture	Guinea pig		Culture	Guinea pig		
		Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate		Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate	Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate
-4	— —	— —	— —	0 0	— —	— —	— —	0 0			
20											
37	+	+	+	0 ↓ +	+	+	+	0 ↓ +	+	+	0 ↓ +

— and + = negative or positive findings, respectively.
0 = no examination made.

? = doubtful.

↓ indicates transfer to another guinea pig.

cated. This experiment was done with cultures in the 60th generation of a strain of murine typhus isolated in September, 1931.

It will be noted that the cultures were no longer viable or infectious after being stored for 10 days at -4°C. or 4 weeks at 20°C. , whereas those kept in the 37°C. incubator were viable and virulent for at least 12 weeks.

In Table II are given the comparative results at 37°C. and at very low temperatures maintained in a freezing box at -20°C. to -25°C.

Preliminary experiments had shown that the cultures could be kept at such low temperatures quite as satisfactorily as at 37°C. Therefore, tests were not made in these particular experiments until the cultures had been stored for several months. The results from three separate experiments are given in Table II. At the end of 10 weeks

TABLE II

Survival of Typhus Fever Rickettsiae in Cultures (53rd Generation) Stored at -20° to -25°C. and 37°C.

	Freezing box (-20° to -25°C.)							37°C.										
	10 wks.				16 wks.			10 wks.				16 wks.				32 wks.		
	Guinea pig			Culture	Guinea pig			Guinea pig				Guinea pig				Guinea pig		
	Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate		Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate	Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate	Culture	Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate	Culture	Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate
Experiment 1	+	+	+	+	+	+	0	+	+	+	0	+	+	+	0	+	+	+
			↓				↓				↓				↓			
			+		-	-	0			+		-	-	-	0			
Experiment 2	+	+	+	0	?	+	+	0	+	+	+	0	-	-	-	0		
							↓			↓								
							0			-								
										+								
	+	+	+	0														
			↓															
			+															
Experiment 3	+	+	+	0	+			+	+	?	0	-	-	-	0			
			↓								↓							
			?								+							
			↓															
			+															

the cultures in each experiment were found to be viable and virulent at both temperatures, whereas storage for 16 weeks was not entirely satisfactory at either temperature. In Experiment 1 for instance, there was no difficulty in recovering cultures from material stored for 16 weeks at either temperature, but there seemed to be some loss in

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virulence since the animals showed a milder infection which was not transferable in the single attempts made after the symptoms had subsided. In Experiment 2 the same results were obtained with cultures stored for 16 weeks at -20°C . but the tests were entirely negative on similar material kept at 37°C . for the same length of time. In Experiment 3 cultures were similarly recovered after 16 weeks at -20°C . but not at 37°C . On the other hand, attention may be called to the fact that (in Experiment 1) cultures kept for 8 months at 37°C . were

TABLE III
Survival of Typhus Fever Rickettsiae in First Generation Cultures and in Infected Tissues Stored at 37°C .

	8 wks.				10 wks.				15 wks.			
	Culture	Guinea pig			Culture	Guinea pig			Culture	Guinea pig		
		Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate		Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate		Febrile reaction	Scrotal lesion	Rickettsiae in scrotal exudate
First generation cultures, kept at 37°C .	+	+	+	0	+	+	+	+	+	+	+	+
Infected tunica minced and suspended in serum-Tyrode mixture, kept at 37°C .						+	+	+		+	-	0

* Blood culture showed a secondary infection. Scrotal lesions were typical of typhus and slides showed rickettsiae only.

found to be viable, and a portion of the material inoculated into a guinea pig gave rise to an infection with typical scrotal swelling.

Many experiments of this kind have been performed in the course of this work, but only a few, which are typical of the results as a whole, are presented in detail. All of 13 experiments at -4°C . were entirely negative in that cultures could never be recovered and characteristic infections in guinea pigs were never obtained with cultures stored for only 10 days. Of 20 experiments at 20°C ., 3 out of 5 were positive after 3 weeks' storage, 1 out of 12 after 4 weeks' storage and none

out of 3 after 5 weeks. At 37° and -20°C. the results were not always as uniform, some cultures kept at 37° surviving longer than others of the same series at -20°, and *vice versa*, but in general, cultures survived at both these temperatures for several months as compared to several days or weeks at the intermediate temperatures of -4° and 20°C. respectively, so that cultures were routinely stored at 37° and -20° during two successive summers for 10 to 12 week intervals and easily recovered with the first transfers thereafter.

To determine whether the results of the above experiments were influenced by a possible adaptation of the rickettsiae to culture conditions, similar tests were made on first generation cultures and on infected tunica suspended in the serum-Tyrode mixture, the flasks for both experiments being kept unopened at 37°C. for the intervals given in Table III. At the expiration of the stated intervals, the cultures in the first generation series were transferred, some of the material from each flask being injected into guinea pigs for virulence. Excellent cultures were obtained from those stored for 15 weeks, as well as for 8 and 10 weeks. The guinea pigs all showed typical typhus infection and rickettsiae were found in tunica scrapings when such examinations were made. The flasks in the series with infected tunica were tested by injecting guinea pigs. After 10 weeks' storage, such material produced a typical infection with a temperature of 40.2° on the 6th day, accompanied by intense scrotal swelling, the slides from the exudate showing rickettsiae. This method of preserving the infectiousness of tissues from typhus-infected guinea pigs would thus seem to be as satisfactory if not better than that of freezing and storing the whole brain or spleen in stoppered bottles at -20°C., which has been used in this and other laboratories. No systematic comparison between these two methods has been made, but it would seem that the former may be preferable, especially in the case of the murine typhus.

DISCUSSION

Earlier attempts to preserve the infectiousness of blood and tissues from typhus-infected animals were attended with variable success. Landsteiner and Hausmann (10) found that emulsions of brain from typhus-infected guinea pigs remained virulent for at least 6 days at 0°C. and 7 days if kept frozen. Olitsky (11, 12) found that blood from

typhus-infected guinea pigs remained infectious only 24 hours at 37°C. but such blood added to a variety of media and kept at 37°C. under aerobic conditions remained infectious for 5 days, while glycerolated brain was no longer infectious after 7 days at 6°C. Hoshizaki (13) found that the blood from typhus-infected guinea pigs remained virulent for 18 days at 4-8°C., 2 days at -15°C. and 7 days at a room temperature of 14-18°C. Laigret and Durand (14, 15) report that the brain of typhus-infected animals remains infectious for at least 20 days when kept frozen at -12° to -15°C. stating that the virus dies within a few hours at ordinary temperature and within 2 to 3 days at 0°C. Kemp (16) found that tissues from typhus-infected animals were no longer active when stored in glycerol at 9°C. for 2 weeks. Bruynoghe and Jadin (17) showed that typhus virus in brain tissue of rats caught on boats in Antwerp could be preserved in the ice box at 4°C. for approximately 1 week.

As has already been pointed out, the preservation of virulence and viability of the cultures for many weeks as described in this paper was in all probability not due to prolonged cultivation *in vitro*, as was shown by the observation that infected tissue (tunica) remained virulent for at least 10 weeks at 37° when minced and suspended in the serum-Tyrode mixture in stoppered flasks. Moreover, first generation cultures remained viable and virulent for at least 15 weeks at 37°C. without having been transferred.

It should be emphasized that the culture flasks were closed with paraffined rubber stoppers. This seal was not broken in the cultures which were used in viability experiments, since earlier experience had indicated that an unbroken seal tended to prolong the viability of the cultures. It has been the experience, in the course of this work, that a rapid increase in the pH of the medium occurred as the result of an incomplete seal, or the temporary removal of the stopper. This rise in pH is presumably due in part, at least, to loss of CO₂. Although of first importance, a favorable reaction is probably not the only factor responsible for the prolonged viability of typhus cultures stored at suitable temperatures. Another effect of the paraffined stopper may possibly be to maintain the oxygen tension as established by the living tissue during the early period of incubation, and it would seem worthy of consideration that a broken seal would lead to changes in the oxygen tension which may be deleterious.

It has been stated by a number of investigators that certain viruses remain viable in cultures only as long as the tissue remains viable. Thus Hecke (18) who has reported in detail on the cultivation of the virus of foot and mouth disease, states that the death of the tissue in the medium is accompanied by rapid destruction of virus, adding that tissue necrosis is well known to cause the destruction of virus. Hallauer (19) found that the titer of his cultures of chicken plague dropped rapidly after the 4th day of incubation. He considered this to be due to the susceptibility of the brain tissue to the virus, the latter increasing rapidly to a concentration which caused the death of the tissue and with it that of the virus. Haagen and Theiler (20) express the opinion, as the result of their work on the cultivation of the virus of yellow fever, that this virus also requires the presence of living tissue in order to remain viable itself *in vitro*. These authors also report a drop in titer beginning with the 4th or 5th day of incubation. Concerning the behavior of typhus fever rickettsiae in culture, Pinkerton and Hass (6) state that the rickettsiae seem to require for their propagation and survival, cells in which metabolism is taking place. They observed that the organisms disappear in less than 1 week when the cells undergo degeneration.

One may assume that in the experiments described in this paper the prolonged viability of the organisms was not dependent on that of the tissue once multiplication had taken place, for it would not seem possible that tissue could remain alive for several months at 37°C. in the medium described. It has previously (3) been pointed out that the rickettsiae are similar to viruses in general in that they also seem to require living tissue for their multiplication *in vitro*. Whether the preservation of cultures of filterable viruses under conditions similar to those suitable for rickettsiae would be possible requires further study.

Pinkerton and Hass (6) state that rickettsiae disappear in successive transplants more rapidly from cultures kept at 37.5° than at 32°. An explanation for the apparent discrepancy between our results and those of Pinkerton at 37° may lie in the different cultural conditions involved, the latter using growing tissue imbedded in coagulated plasma. We have noted that the acidity produced in such cultures is considerably retarded at 32° as compared with 37°.

So far, no explanation can be offered for the peculiar differences in the results conditioned by the various temperatures. It is not clear why the viability of the cultures is maintained for long periods at 37° and -20° but not at the intermediate temperatures of 20° and -4° , the other conditions being identical. That organisms can remain viable for a long time at very low temperatures probably finds explanation in the slowing down of metabolic activity, but it is difficult to find an explanation for the fact that the same organism survives for a very much longer time at 37° than at 20° , and longer at 20° than at -4° .

With regard to the etiological significance of the cultures kept at fever, it is noteworthy that the infectiousness of the cultures kept at the various temperatures for varying periods of time seemed in general to run parallel to the number of viable rickettsiae in the cultures. Moreover, the 61st generation of a strain which had been carried in cultures for over $2\frac{1}{2}$ years, transfers always being made with material selected for its abundance of rickettsiae, gave rise to characteristic typhus infection in guinea pigs and typical Weil-Felix reaction when injected into a rabbit.

SUMMARY

1. Cultures of typhus fever rickettsiae were as a rule found to remain viable and virulent after being stored at 37° and -20°C. for several months, whereas they failed to survive when stored at the intermediate temperatures of 20° and -4°C. for 4 weeks and 10 days respectively. In one instance cultures were stored at 37°C. for 8 months without having been transferred, and were subsequently found to be viable and infectious.
2. The conditions influencing such long survival of an organism which seems to require living tissue for multiplication (as filterable viruses do in general) are discussed.
3. Typhus-infected tissues (minced guinea pig tunica) suspended in a serum-Tyrod mixture in sealed flasks remained infectious at 37°C. for at least 10 weeks.
4. Additional evidence for the etiological significance of the rickettsiae in typhus fever is obtained from the experiments described.

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PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

X. CHEMICAL PROPERTIES OF CHICKEN TUMOR EXTRACTS

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The present investigation has to do with the physical and chemical properties of active extracts prepared from chicken tumor tissues, on the assumption that the neoplastic principle may exist in solutions, combined with a certain number of normal or pathological products. The first group of observations to be described have been briefly reported in previous communications (1). More attention is given here to the chemical results. In the following paper, methods for further purification of the active extracts are reported. The purification of the tumor extracts so far obtained is presented as an introduction to the study of the chemical characteristics of the specific agent.

Until recently few attempts had been made to study the properties of the active tumor extracts by direct chemical means. Sugiura and Benedict (2) found that half saturation with ammonium sulfate produced a precipitate in Berkefeld filtrates of chicken tumor material. The activity of the filtrate appeared to go with the precipitate, which they designated as the "globulin" fraction, while the "albumin" fraction was inactive. In this laboratory, fractional precipitation was secured by means of electro dialysis with the tumor-producing activity definitely going with the precipitate (3). The precipitation was found to be due to acidification resulting from electro dialysis, not to the reduction in the salt concentration. A similar fraction carrying the activity was produced by bringing the acidity of concentrated Berkefeld filtrates to or beyond pH 4.8. Below pH 4.0 both fractions are inactivated. A less clear-cut separation of the agent with a "globulin" fraction has been also reported by Fränkel

(4), who induced an acid reaction in tumor extracts by bubbling through carbon dioxide. However, we have not been able to demonstrate the presence of true globulin in chicken tumor extracts.

Analysis of the active fraction obtained by electro dialysis or by the addition of weak acid gave figures for the nitrogen and phosphorus which did not differ materially from those given by the full tumor extract. Moreover, later work showed that most of the tumor protein could be removed without affecting sensibly the tumor-producing activity.

Source of Material and Preparation of Extracts

The tumor used throughout the studies was the spindle cell sarcoma known as the Rous sarcoma, or Chicken Tumor I of the Rockefeller Institute series. In the preparation of the material great care was taken to free the "healthy" tumor tissue from the necrotic parts and from the surrounding normal and reactive tissue.

Extracts from Fresh Tumor Tissue.—The usual method of extraction is to grind thoroughly with sterile sand 25 gm. of tumor pulp obtained by passing the tissue through a masher and slowly adding 500 cc. of distilled water. The reaction is maintained between pH 7.2 and 7.4 by the use of 0.1 N NaOH. The mixture is carefully shaken to insure homogeneous extraction and the larger particles are removed by centrifugation. This material, sometimes prepared with a different proportion of solvent, will be referred to as tumor extract.

Berkefeld Filtrates.—The tumor extract is filtered through Berkefeld V candles under 30 lb. of air pressure. In a sample experiment the dry weight of 1 cc. of extract was 4.8 mg. while that of the filtrate was 1.9 mg. However, the process of filtration involves appreciable loss in the active agent. For this reason, and in order to overcome the dilution necessary to make possible filtration, we regularly concentrated the filtrate. This was accomplished in Norton alundum thimbles lined with an 8 per cent solution of cotton in glacial acetic acid (5). With reduced air pressure an 8- to 10-fold fluid concentration can be obtained in the course of 2 to 3 hours. The material will be referred to as concentrated filtrate.

Extracts from Desiccated Tumor Tissue.—Murphy (6) has shown that it is possible to preserve the tumor agent over long periods in desiccated tissue, provided the desiccation is brought about rapidly at a low temperature. The tumor pulp is placed in vacuum jars with sulfuric acid as dehydrating agent and desiccation allowed to take place in the ice box. The great variability of filters and the frequency with which filtrates of low potency are encountered has led to the use of extracts from desiccated material whenever this is possible. As routine, a number of powdered desiccates are pooled, sifted through a fine mesh, thoroughly mixed, and the grade of activity tested in advance by inoculation into chickens. The preparation is then stored in sealed tubes. Thus sufficient material of known activity may be secured for a number of months of experimentation. It is es-

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timated that 1 gm. of the desiccate corresponds to about 8.5 gm. of fresh tissue, but the dry weight may vary greatly with each individual tumor.

For the preparation of the extract 1 gm. of the powdered desiccate is extracted with 60 cc. of distilled water. During the extraction the reaction is adjusted to pH 7.4 by the addition of 0.1 N NaOH. The debris is removed by centrifugation and the supernatant fluid filtered through coarse paper.

General Properties of Active Tumor Extracts

The general characteristics of the water extracts of desiccated tumors differ little from those of concentrated Berkefeld filtrates. The former material is always opalescent while the latter is clear. Opalescence goes with non-filtration. However, this character does not seem to affect greatly the final composition of the solutions.

TABLE I
Solubility of Chicken Tumor Material in Various Solvents

Solvent.....	Nitrogen per cc.			
	H ₂ O	H ₂ O, pH 7.6	Ringer's solution	5 per cent NaCl
	mg.	mg.	mg.	mg.
From fresh tissue.....	0.442	0.592	0.447	0.661
From desiccated material.....	0.743	0.873	1.025	1.066

* Average from six experiments.

The extract prepared according to the present method is always a mucoid fluid, more or less colored by blood pigment derivatives and the high viscosity is typical of Chicken Tumor I products.

The material going into solution varies with the methods used, but within certain limits the differences are mainly quantitative. Table I gives a summary of the nitrogen content of extracts prepared with distilled water, alkaline water (the reaction being maintained around pH 7.6 by means of 0.1 N NaOH), Ringer's solution, and 5 per cent sodium chloride solution.

Tumor extracts subjected to increasing acidity show gradual development of opalescence and finally a precipitate separates when the reaction of the fluid attains pH 4.8 or drops below that point. Such precipitation can be obtained by any means which provides the

adequate acidity. If an excess of acid is added at once, a mucoid precipitate forms, which contracts, clearing the fluid of most of the dissolved material. Better fractionation is obtained when buffer solutions of a convenient pH are used. In this case, the presence of the salt itself has a definite action on the physical properties of the precipitate, which becomes flocculent; and substances which otherwise would be absorbed remain in solution.

The main part of the fraction carried down by acid precipitation has the physical characters and the chemical composition of a mucoprotein. This protein fraction can be brought again into solution upon treatment with alkali. Salt, for example NaCl or sodium acetate, also increases the solubility of chicken tumor extracts, which lose their typical mucoid characteristics. Neutral or alkaline filtrates of fresh tumor tissue are not coagulated by heat and they retain their original limpidity. If salt is present a slight opalescence develops, but no actual precipitation is obtained on boiling.

The chicken tumor agent will stand high salt concentration, from 1 per cent NaCl to half saturation with ammonium sulfate, which gives rise to precipitation without inactivation. The agent generally goes with any precipitate produced in the fluid if precautions are not taken, doubtless as the result of adsorption. This adsorption is apparently not specific. In case of acid fractionation the activity is associated with the precipitate, except when the reaction has been allowed to drop below pH 4.0, at which point the agent is regularly inactivated.

Purification of active extracts obtained in these ways has been continued, but as yet no attempts have been made to isolate the agent itself by direct chemical means. The following methods were devised for the successive elimination of inactive contaminating fractions, the volume of the original solution being maintained practically constant. The first practical step in purification was the separation of the agent from the major part of the chicken tumor proteins.

Purification by Adsorption

Leitch first applied the method of adsorption and elution to the study of chicken tumor material (7). Other investigations have shown that a variety of substances can adsorb or inactivate the chicken tumor agent (8). Kaolin, alumina gel, and charcoal have

been studied in this respect (Fränkel, Lewis, Maschmann) and in several instances the agent has been partially released in active form from its union with the adsorbent. However, the method widely applied by Willstätter to the concentration of enzymes failed with the chicken tumor agent in that no enrichment of the active principle could be obtained by elution.

Investigating the possibilities of the adsorption method for purification we became interested in the fact that, when using a proper ratio of adsorbent to extract, most of the activity would remain in the solution, whereas the major part of the contaminating substances were removed during the process of adsorption. The purified extract appeared to deserve more attention than the usual eluate from the point of view of isolation of the active principle, and the properties of this fraction have been carefully considered.¹

Adsorbent.—Aluminum hydroxide, prepared according to Willstätter and Kraut's directions for the Type C was first used (9). This preparation, however, leaves a turbid fluid when the mass of the colloid is removed. Later we resorted to a modification of this type with the advantage of obtaining a clear supernatant fluid, free from suspended aluminum hydroxide particles and suitable for experiments involving quantitative analysis.

The technique and proportions are those used for the preparation of regular Willstätter Type C, i.e. 30 gm. ammonium sulfate and 50 gm. aluminum sulfate for a 4 liter preparation with the difference that 7.4 gm. and 1.2 gm. NH_3 are used instead of 7.75 gm. and 1.47 gm. respectively. After the second addition of ammonia the washing with distilled water is repeated until the supernatant fluid which separates on standing becomes sulfate-free. This is obtained after about 10 to 12 washings. In this case the water of suspension remains perfectly clear and never becomes turbid, even if washing is continued indefinitely.

Following the last washing the preparation is allowed to settle and the supernatant water discarded until the material has been concentrated to 850 cc. total volume. This standard concentrated suspension constitutes our stock preparation. The gel under these conditions is a voluminous and flocculent material, with a bluish, transparent appearance. The reaction is slightly acid, an important condition, for a similar adsorbent having an alkaline reaction exhibits but a slight affinity for the chicken tumor proteins.

Desiccated at 105°C. the concentrated preparation leaves a dry residue of 13.0 mg. per cc. This material is not represented entirely by pure aluminum hydroxide, but sulfate ions are also retained on the colloid molecule. Additional wash-

¹ This part of the work was carried out in collaboration with Dr. O. M. Helmer.

ings with distilled water do not affect the composition of the product. Other attempts to rid the gel of the associated sulfate group have failed, as its removal changes the properties and the adsorbing power of the gel. The amount of bound sulfate corresponds to about 0.60 mg. SO_4 per cc.

During the process of adsorption there is an exchange of ions taking place, probably with the free phosphate radicals, while appreciable amounts of sulfate go into solution. This fact has no practical bearing on the results except when total sulfur analysis is involved, in which case dialysis or barium precipitation may be resorted to.

Technique of Adsorption.—In the procedure of adsorption, the proportion of adsorbent to tumor extract has been kept constant, one volume of concentrated filtrate or of extract from desiccate being treated with an equal volume of our concentrated aluminum hydroxide suspension. Prior to use, the aluminum hydroxide is first centrifuged for a short time, avoiding the formation of a too compact deposit, and the excess of water is discarded. This precaution was found necessary in order to avoid dilution of the active material, which would have disturbed quantitative determination with respect to the chemistry and potency of purified extracts. The gelatinous deposit is added to the tumor extract and shaken together until a homogeneous mixture is obtained. The adsorbed material is spun down and the supernatant fluid collected by decantation and filtered through coarse paper.

Washing of Adsorbate.—The deposit collected after adsorption is washed with distilled water and the washings are repeated as many times as may be required to rid the gel of all the non-adsorbed but adherent material. The last washings which prove to be clear and colorless are as a rule devoid of any tumor-producing activity and respond negatively to the usual color tests. For the present series of experiments analysis of this fluid gave average figures amounting to 0.005 mg. nitrogen and 0.023 mg. reducing substances per cc.

Elution.—The alkaline agent used for elution has been mainly dibasic sodium phosphate at 0.07 M concentration or more often as a 1 per cent solution. The washed deposit was extracted by shaking for 3 minutes with a volume of phosphate solution equal to the volume of the original extract. After centrifugation the released fraction was filtered through coarse paper. The material obtained by elution generally carries over the color of the first tumor extract. The biuret, Millon, ninhydrin tests, and the diazo reaction are positive. An abundant precipitate is obtained at pH 4.7 by the addition of acetic acid. On analysis the figures obtained are as high as 0.147 mg. nitrogen and 0.161 mg. reducing substance per cc. The tumor-producing activity of this material is about 50 per cent of that of the crude extract. As this step does not constitute an improvement as regards the purification of the agent, this fraction has not been included in the present study. The properties of the other extracts will be considered separately.

Nature and Chemical Composition of the Tumor Extracts

Plain Tumor Extracts

In addition to the general properties of the concentrated filtrates and extracts from desiccates which have been described above, chemical analysis has shown an average of 0.474 mg. nitrogen per cc. in the concentrated filtrate and 0.680 mg. nitrogen per cc. in the dry powder extracts. The color tests for proteins are regularly positive. No free reducing sugars are found, but the Molisch test and the naphthoresorcin test for glucuronic acid are positive. On acid hydrolysis the test with Fehling's solution becomes strongly positive. Moreover, the tumor extracts yield large amounts of reducing substances which, figured as glucose, correspond to an average value of 0.452 mg. per cc. for concentrated filtrates and 0.638 mg. per cc. for desiccated extracts.

These results, along with the typical physical characteristics of the tumor extracts, certain color reactions, and the fact that as much as 2.4 per cent sulfur was obtained from the precipitated protein, indicate that the tumor material is represented to a large extent by a mucoprotein.

The nitrogen determinations in connection with this work were made according to the Van Slyke micro Kjeldahl method (10). The method of Van Slyke and Hawkins (11) was used for the determination of reducing sugars. In the latter case the solutions were first acidified with hydrochloric acid to a 10 per cent final concentration and the material hydrolyzed by 3 hours' immersion in boiling water.

Extracts Purified by Adsorption

The process of adsorption eliminates from the solution the pigments which are normally present in chicken tumor extracts. When the starting material is a Berkefeld filtrate the supernatant fluid, after removal of the aluminum hydroxide, is a clear, colorless fluid with a high viscosity. If an extract from a tumor desiccate is the source the fluid is also colorless, and equally viscous, but remains opalescent. The solid content of such purified extracts after evaporation and desiccation *in vacuo* is about 2 mg. per cc.

The usual color reactions for proteins, for example the biuret, Millon, and xanthoproteic tests, are negative. No precipitate is produced in the aluminum supernatant by the addition of tungstic acid, picric acid, or trichloroacetic acid. The addition of tannic acid fails to produce any apparent modification of the fluid, except for a marked increase in the original viscosity. On chemical analysis the average figures for nitrogen are 0.038 mg. per cc. in the case of concentrated filtrates and 0.076 mg. per cc. in the case of desiccate extracts.

These results show that the process of adsorption has removed the bulk of the proteins and the supernatant fluid has retained only 8 to 11 per cent of the total nitrogen originally present in the plain tumor extract.

The aluminum supernatant has no reducing power on Fehling's solution. After acid hydrolysis, however, reducing substances appear and the test becomes strongly positive. The Molisch test is definitely positive with characteristic color rings. Positive naphthoresorcin reaction is suggestive of the presence of glucuronic acid. As in the case of the crude extracts it is found that acid hydrolysis liberates large amounts of reducing substances in the purified fraction. Calculated as glucose, the yield was 0.098 and 0.138 mg. per cc. respectively, whether fresh filtrate or tumor desiccate was used for the preparation of the solutions. These values correspond to 21.6 per cent of the reducing substances originally found in the plain extract. Moreover, the aluminum supernatant has a content of about 0.011 mg. non-diffusible sulfur per cc., or more than 3.0 per cent as figured for the dialyzed material. However, the sodium nitroprusside test for $-SH$ groups and the Schiff reaction for aldehyde are negative. The bound sulfur is easily released by acid hydrolysis.

These results and the study of other chemical tests support the view that the main component present in the fluid after adsorption is a carbohydrate; namely, chondroitin-sulfuric acid. Moreover, the high viscosity already noted with crude extracts and especially characteristic of the aluminum supernatant fluid can be ascribed directly to the physical state of the carbohydrate itself. The viscosity of the material is rapidly lost on acid hydrolysis.

Other observations are in line with the above mentioned conclusions.

The solubility of this fraction is not affected by dilute acetic acid or buffered solutions of higher hydrogen ion concentration. The addition of 1 or 2 volumes of glacial acetic acid produces the separation of the substance as a white, flocculent material. Treatment of the aluminum supernatant with 2 volumes of acetone, 95 per cent alcohol, or with an alcohol-ether mixture has the same effect.

Crystalline hexosamines have been recovered and an osazone obtained from the isolated material after treatment with strong acid.

Tumor-Producing Agent in Aluminum Supernatant Fluid.—The results as concerns the tumor-producing activity of the extracts so far studied have been analyzed in other publications (12). It was found that, allowing for the effect of the removal of an inhibiting

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factor, the fraction retained practically the same tumor activity as the crude extract. This is only true in the limits given by the sensitivity of the test, since an unknown portion of the activity was found to go with the adsorbent. However, this portion has been con-

TABLE II
Aluminum Hydroxide Adsorption Experiments
Comparative Analysis of the Different Fractions for Nitrogen and Reducing Substances

Experiment No.	Nitrogen per cc.				Reducing substances Glucose per cc.				Index of tumor-producing activity			
	Concentrated filtrate	Supernatant fluid	Wash water	Eluate	Concentrated filtrate	Supernatant fluid	Wash water	Eluate	Concentrated filtrate	Supernatant fluid	Wash water*	Eluate
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.				
1	0.398	0.029	0.003	0.060	0.455	0.080	0.007	0.124	0	0	0	0
2	0.647	0.039	0.006	0.220	0.473	0.064	0.003	0.214	66	66	0	33
3	0.376	0.028	0.003	0.049	0.223	0.036	0.004	0.060	100	100	0	50
4	0.516	0.030	0.001	0.095	0.294	0.076	0.002	0.149	66	72	0	16
5	0.436	0.040	0.005	0.066	0.375	0.088	0.004	0.090	100	100	0	60
6	0.316	0.061	0.006	0.325	0.680	0.196	0.044	0.232	100	100	100	100
7	0.629	0.044	0.006	0.148	0.665	0.148	0.007	0.170	100	100	0	100
Average....	0.474	0.038	0.004	0.137	0.452	0.098	0.010	0.148	88.6	89.8	—	51.0
Extracts from desiccates												
8	0.687	0.061	0.005	0.163	0.588	0.082	0.024	0.120	100	100	0	50
9	0.528	0.053	0.006	0.103	0.588	0.072	0.008	0.076	66	66	0	0
10	0.745	0.063	0.005	0.217	0.750	0.177	0.050	0.282	100	100	50	100
11	0.652	0.096	0.007	0.107	0.440		0.032	0.120	50	50	10	50
12	0.796	0.107	0.005	0.199	0.826	0.224	0.058	0.278	100	100	50	80
Average....	0.681	0.076	0.005	0.158	0.638	0.138	0.034	0.175	83	83	22	56

* Tumors obtained from the wash water should be ascribed to an incomplete preliminary washing of the adsorbate.

sidered negligible, the tumors resulting from inoculation of the purified fraction, as compared with those produced by the original extract, giving an equal percentage of takes and having at least an equivalent rate of growth.

Tables II, III, and IV include a comparative study of the chemical

content of the active extracts, with an index of the tumor-producing activity.

In addition to the fact that the main tests for proteins have become negative, 92.0 per cent of the nitrogen and 78.4 per cent of the reduc-

TABLE III

*Results from Analysis and Inoculation Tests of the Tumor Extracts before and after Adsorption**

	With concentrated filtrate			With desiccate extracts		
	Nitro- gen	Reduc- ing sugars	Tumor- produc- ing activity	Nitro- gen	Reduc- ing sugars	Tumor- produc- ing activity
	per cent	per cent	per cent	per cent	per cent	per cent
Tumor extracts.....	100.0	100.0	100.0	100.0	100.0	100.0
Aluminum supernatant fluid.....	8.0	21.6	101.3	11.1	21.6	100.0
Amount removed by adsorption.....	92.0	78.4	0.0	88.9	78.4	0.0

* Figures calculated on the basis of the original extracts as 100.

TABLE IV

Color Tests on Chicken Tumor Extracts before and after Adsorption with Aluminum Hydroxide

Reaction	Tumor extract	Aluminum supernatant
Biuret.....	++++	—
Xanthoproteic reaction.....	+	—
	(but not typical)	
Millon's.....	+++	—
Molisch's.....	++++	++++
Naphthoresorcinol.....	++++	++++
Schiff's (for nucleic acid).....	++	—
Sodium nitroprusside (for —SH).....	—	—
Ninhydrin.....	++++	+++
Diazo reaction.....	++++	+++

ing substances have been discarded as inactive, contaminating products. However, the ninhydrin test and the diazo reaction remain strongly positive.

Additional Properties of the Aluminum Supernatant Fluid.—The next point in the investigation was to establish the relationship which

would exist between the carbohydrate present in the active extract and the tumor-producing activity itself. Many reagents were tested and a number were found to precipitate the carbohydrate from the fluid.

The more characteristic of these reagents were a certain number of basic dyes, especially safranin and neutral red. When some of a 0.5 per cent aqueous solution of neutral red is added to the aluminum supernatant, the viscosity first increases and the fluid becomes gelatinous. On shaking, the precipitate contracts and separates as a mucoid material floating on the surface, leaving a fluid, clear and deprived of viscosity. The presence of salts changes the character of the precipitate. In 1 per cent NaCl the precipitate becomes flocculent, while in 3 per cent salt the compound is completely soluble. The latter property was used for the redissolving of the dye-carbohydrate fraction when the purification of the aluminum supernatant was attempted by this method. Analyses for reducing substances were as follows: aluminum supernatant: 0.173 mg. glucose per cc.; neutral red precipitate, washed and redissolved: 0.136 mg. glucose per cc. The chicken tumor activity was lost during the procedure, not as the result of a direct toxic effect of the dye which from other tests had been found to be innocuous to the agent, but probably owing to the physical character of the precipitation itself. Nevertheless, the neutral red reaction has been found very useful and is used in the laboratory as a routine test for the detection of the carbohydrate in chicken tumor extracts.

Certain salts of heavy metals, such as silver nitrate, mercuric acetate, ferric chloride, gold chloride, precipitate the aluminum supernatant, but only when the acidity has dropped below pH 4.0 by the decomposition of the chemicals and liberation of strong acid. At that point the chicken tumor agent is inactivated. A certain fraction of the purified extract precipitates out on addition of basic lead acetate, with the reaction remaining around neutrality. Precipitation occurs with uranium acetate and copper acetate with the reaction at pH 6.0. In this case the precipitation is incomplete or the excess of the reagent induces an acute reaction in the host.

Purification of the Aluminum Supernatant Fluid by Precipitation with Gelatin

The properties and chemical reactions shown by the aluminum supernatant fluid demonstrate that the protein content is relatively low and that the constituent of next importance is a carbohydrate, mucoitin- or chondroitin-sulfuric acid. Additional purification by means of ordinary precipitating reagents could not be accomplished on account of the immediate destruction of the agent following the use of chemicals giving insoluble combinations with the carbohydrate.

A method was then devised for the removal of this compound with a basic protein. The method originated in the fact that the tumor carbohydrate has a slightly acid character and may pre-exist normally as the prosthetic group of chicken tumor mucoproteins. Gelatin was selected on account of favorable chemical properties and because of a certain hypothetical genetic relationship which it was believed exists between the two compounds in the connective tissues.

It was found by preliminary tests that when gelatin is added to the aluminum supernatant fluid, and the reaction adjusted at or below pH 4.8, precipitation is induced in the mixture. No precipitation occurs in either the aluminum supernatant or the gelatin solution alone on acidification. Likewise, there is no apparent reaction taking place between the chicken tumor material and the gelatin when mixed together.

If enough acetic acid is added at once, a mucoid precipitate develops in the mixture, similar in appearance to that obtained from an ordinary mucoprotein solution. Very dilute acetic acid added progressively, acetic acid in presence of salts, or buffered solutions at a convenient pH, bring about a flocculent precipitate provided the acidity reaches at least pH 4.8. The optimal conditions for the precipitation are at pH 4.7, at which point the precipitation is complete while the corresponding acidity has no harmful effect on the agent.

For the present investigation our source of material has been extracts prepared from desiccated tumor tissue only, and the adsorption and removal of the proteins has been performed in the usual manner.

To 10 cc. of the aluminum supernatant fluid is added 1 cc. of a 2 per cent commercial gelatin solution (Gold Label). The reaction is brought to pH 4.7 by means of 0.1 M acetate solution at pH 4.6 (usually 2 cc.) and the reagents mixed together. On standing for 10 to 20 minutes, a voluminous, flocculent precipitate forms and separates slowly. The precipitate is removed by centrifugation and the gelatin supernatant filtered through course paper. The gelatin precipitate is washed several times in 0.02 M acetate buffer at pH 4.6 and redissolved in a sufficient amount of Ringer's solution to bring the volume back to that of the original extract.

Gelatin Supernatant Fluid.—The material recovered after gelatin precipitation is a water-clear, colorless fluid, which is devoid of the usual viscosity of active tumor extracts. Qualitative tests indicate that the carbohydrate retained in abundance in the aluminum supernatant has been eliminated by the isoelectric precipitation of the new gelatin compound. The gelatin supernatant is no longer precipitated by basic dyes, basic lead acetate, mercuric acetate, or Millon's reagent. The naphthorescorcin test for glucuronic acid is negative while strongly positive on the gelatin precipitate.

Gelatin Precipitate.—Redissolved in Ringer's solution or in slightly alkaline solution the gelatin precipitate gives an opalescent and viscous solution, very similar in appearance to the original aluminum supernatant fluid. From the fluid, material can be extracted which, in water, has the typical characteristics of the substance found in active aluminum supernatant fluid. It is precipitated by basic dyes, as usual, by basic lead acetate, and also by acetone and dilute alcohol. The products from gelatin precipitation were tested with respect to their ability to induce tumors. The gelatin supernatant was found to have a tumor-producing activity estimated at 69 per cent of that of the aluminum supernatant. The tumors obtained were

TABLE V
Fractionation by Gelatin

	Aluminum supernatant fluid	Gelatin supernatant fluid	Gelatin precipitate
Precipitation with neutral red.....	+++	—	+++
Precipitation with basic lead acetate.....	+++	—	+++
Precipitation with Millon's reagent.....	++	—	++
Test for glucuronic acid.....	++	—	++
Index of tumor-producing activity.....	91.7	69.2	43.3

usually slightly larger than those of the control.² The dilution to which the gelatin supernatant was subjected through the manipulations was not allowed for in estimating the results. The gelatin precipitate yielded an average of 52 per cent of takes and the tumors were somewhat smaller than those produced by the control material. In a few experiments the gelatin supernatant proved to be as active as the control extract while the gelatin precipitate was inactive. Table V gives the general properties of the "gelatin fractions," together with an index of their tumor-producing activity.

In dealing with the gelatin method one may encounter more difficulties than with the first step of purification by adsorption. One may have to get acquainted with the physical peculiarities of the precipitation and adapt the amount of gelatin to the concentration and

² Gelatin mixed with active material seems to enhance slightly the development of the resulting tumor. This effect may account for the large tumors obtained from the fraction contaminated by an excess of gelatin.

viscosity of the tumor extract. With some experience the adsorption of the active agent on the gelatin precipitate can be easily avoided.

The results reported indicate that a material remaining after adsorption on aluminum hydroxide and representing about one-third of the total solids found in active aluminum supernatant fluids is eliminated by the gelatin method. This substance which is present in all chicken tumor extracts is not connected with the tumor-producing activity.

SUMMARY

A brief description is given of the main physical and chemical characteristics of active chicken tumor extracts. By special methods constituents have been removed from the active solution: (1) the major part of the tumor-proteins, which are adsorbed on alumina gel and discarded; (2) a carbohydrate, present in large amounts in the tumor extract, is eliminated by precipitation in combination with gelatin. In both cases the tumor principle is not directly affected and largely remains in active form in the solution.

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PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

XI. CHEMICAL COMPOSITION OF PURIFIED CHICKEN TUMOR EXTRACTS CONTAINING THE ACTIVE PRINCIPLE

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Previous work has shown that the tumor-producing agent found in cell-free extracts of Chicken Tumor I can be separated from most of the contaminating proteins by means of adsorption. A further step in the separation of the agent was obtained by the removal of a carbohydrate present in large amounts in all chicken tumor preparations. This was accomplished by combining the carbohydrate with gelatin and precipitating the compound at its isoelectric point (1). The further purification by elimination of inactive products has been continued. The present report deals with the effects of dialysis upon the chemical composition and the tumor-producing activity of chicken tumor extracts.

The spontaneous inactivation of the agent while in solution, especially rapid at room temperature, requires that a method be adopted which will accomplish the purification yet preserve the activity of the fluid. This condition was practically attained by performing all the manipulations, from the preparation of the extract to the inoculation tests, in the course of the same day.

Materials and Methods

Preparation of Water Extracts.—The preparation of active extracts from desiccated tumor tissue has been described in the previous article (1). For the present investigation Berkefeld filtrates were not used, since the potency of extracts is usually considerably reduced by filtration. The purification of fresh tumor extracts by direct adsorption of large amounts of inactive material on aluminum hydroxide has provided a method which offers products of greater purity without any apparent loss in tumor-producing activity. The tumor extracts which have been used almost exclusively in the course of this study were prepared as follows:

The tumor pulp obtained in the usual way (1) was ground with sterile sand into a homogeneous paste. The sand was ordinary beach sand repeatedly treated with dilute nitric acid and washed with distilled water. Care was taken that before use the sand be acid-free, since any important shift in the hydrogen ion concentration of the tumor extract on either side of the neutral point may greatly affect the condition of the subsequent adsorption.

The tumor paste was stirred with cold distilled water which was added in small quantities totalling in volume 12 times the weight of the original tumor pulp. The extraction was finished by shaking the mixture for 2 minutes. The debris was separated by centrifuging the mixture for 10 minutes and then the supernatant fluid was filtered through sterile gauze.

Water preparations obtained in this way have the general characteristics of ordinary extracts of Chicken Tumor I. In the present work no alkali was used in the extraction and the reaction of the fluid remained around pH 7.0 to 7.3.

It was important to obtain extracts free from bacterial contamination, in view of the quantitative chemical analysis. As the filtration through Berkefeld candles was avoided, care was taken to secure sterile tumors as starting material. In this study all fractions obtained from tumor extracts, including the dialyzed solutions, were regularly tested for sterility on broth and agar media. The culture tests were always found to be negative except those of the last dialyzed fraction with which the media sometimes showed occasional isolated cultures after several days incubation.

Adsorption.—The extract of tumor pulp was fractionated by direct treatment with an equal volume of concentrated aluminum hydroxide preparation, as described in the previous paper (1). After centrifugation the supernatant fluid was filtered through coarse paper to insure homogeneity of the solution. This filtrate did not differ in its general characteristics from the aluminum supernatant fluid obtained heretofore from concentrated Berkefeld filtrates or from extracts of tumor desiccates. This fraction was used throughout as the starting material.

Dialysis.—The usual methods of dialysis were too slow to be used in this work, because of the inactivation of the chicken tumor agent, especially rapid in cell-free preparations. A satisfactory method was found by the use of a device adapted by Northrop and Kunitz (2) to hasten diffusion through collodion membranes. With this technique the end-point of dialysis of chicken tumor extracts was reached within a few hours.

Collodion Bags.—The bags were prepared by rotating mechanically for 20 minutes 6 cc. of Merck's U.S.P. collodion (4 per cent by weight in ether-alcohol solution) in 150 x 18 mm. test tubes kept in a nearly horizontal position. The layer of collodion was aerated by the flow of about 500 cc. of air produced by the displacement of an equal volume of water. This current of air was blown gently through a glass tube introduced in the test tube during the last 10 minutes of rotation. The collodion membrane was then washed several times with water, removed from the glass, and tested. These collodion bags can be stored in dis-

tilled water, in the cold room above freezing temperature for several weeks. The capacity of these bags is about 23 cc., with a surface of semipermeable membrane of 2.6 sq. cm. for each cc. of solution. When a greater volume was needed 175 x 22 mm. test tubes were used with 9 cc. collodion solution. There is an advantage in using smaller test tubes, since the ratio of surface of diffusion to the volume is inversely proportional to the radius, and consequently dialysis may be completed more rapidly.

Rocking Apparatus.—The rocking machine used was constructed after Kunitz and Simms (3). Glass stoppers were adjusted to the collodion bags under slight pressure, and held tightly by rubber bands. Air bubbles in the solution to be dialyzed were avoided in order to reduce the possibility of oxidation. The solution was kept agitated during dialysis by the motion of a glass marble which was chosen for the largest size admitting free movement from one end to the other of the collodion bag. During the rocking, distilled water at the desired temperature was made to flow through the dialyzing glass chamber at the rate of 50 cc. per minute.

Time of Dialysis.—Preliminary determination of the variation observed in the total dry weight of the solution has shown that the removal of the diffusible portion from the aluminum supernatant fluid was practically complete after 3 hours of dialysis. After 1 hour of dialysis the content of several sacs in use was combined to ensure homogeneity of the samples taken for chemical analysis and inoculation tests. Dialysis of the remaining fluid was then continued in the same or in new membranes and samples were taken again after periods of 2, 3, and 5 hours.

Chemical Methods.—

Determination of Total Solids.—Extracts both from desiccates and from tumor pulp were submitted to dialysis. The solids content of the different fractions was determined from the total dry weight of 3 cc. and 6 cc. of the water extract and of samples taken after 1, 2, and 3 hours dialysis. The samples were first evaporated to dryness at 90°C. and the residue maintained at 105°C. for 2 hours. The material was then allowed to cool at room temperature in the presence of CaCl_2 and weighed.

Nitrogen Determinations.—These were obtained by the gasometric micro Kjeldahl method of Van Slyke (4), and the *reducing substances* were determined after acid hydrolysis by the method of Van Slyke and Hawkins (5).

Total Sulfur.—This was determined by the method of Benedict (6). Digestion of material was performed more often with the oxidizing mixture of Denis (7), and on two occasions according to the technique of Carius. The barium sulfate precipitate was collected either on asbestos fibers or on the Neubauer microcrucible of Pregl (8).

Total Phosphorus.—In chicken tumor extracts the total phosphorus was estimated as the molybdophosphate by the microgravimetric method of Pregl (8),

or at times by the colorimetric method described by Leiboff (9). The procedure of Van Slyke (4) for micro Kjeldahl digestion was applied to the digestion of the tumor material, except that the sulfuric-phosphoric acid reagent was replaced by a sulfuric-nitric acid mixture. If large amounts of protein were present, oxidation was more easily accomplished by the use of sodium chlorate in place of persulfate. The formation of perchloric acid and the danger of explosion were prevented by dilution of the acid mixture as follows: 1 volume concentrated H_2SO_4 , 2 volumes HNO_3 , and 3 volumes H_2O . This acid mixture was used in the proportion of 1 cc. to 0.5 gm. of chlorate. The samples were pipetted into 200 by 25 mm. test tubes of pyrex glass and evaporated to dryness in the water bath. 1 cc. of water was added to each tube prior to digestion. The digestion was conducted until crystallization of the salt began to take place. Then 5 cc. of water were added and the digestion mixture boiled for 5 minutes. In some instances the material was treated by the Carius method.

Inoculation Tests for Activity.—Samples of the solutions at different stages of dialysis were tested for tumor-producing activity by intracutaneous inoculation into normal chickens of 0.2 cc. of each fraction. The original material, inoculated similarly in the same bird, served as simultaneous control. The size of the skin tumors produced was recorded 2 or 3 weeks after injection, according to individual variations in the rate of growth. The percentage of positive inoculations was taken as another index of the tumor-producing power of the extracts. In the tables these two figures have been combined in order to obtain a single index of potency. These figures are not intended to give an accurate measure of the actual content of active principle in the extracts, but so far it seems to be a close estimation of the actual ability of different solutions to induce tumors.

RESULTS

Effect of Dialysis upon the Total Dry Weight and the Tumor-Producing Activity of Chicken Tumor Extracts

Adsorption to aluminum hydroxide of a large portion of the inactive constituents of the tumor extracts had already been shown. According to the present data, about 74 per cent by weight of the dry material had been eliminated in this manner. Furthermore, 73.2 per cent of the aluminum supernatant fluid was found to consist of diffusible products.

Figures given in Table I show that the diffusion was rapid: of the diffusible substances four-fifths escaped during the 1st hour and practically all within 3 hours. This treatment reduced the dry weight to 0.43 mg. per cc. of dialyzed extract, with upper and lower limits of 0.61 and 0.16 mg. per cc., compared with 1.60 mg. per cc. for the aluminum supernatant fluid and 6.14 mg. per cc. for the water extract.

As this investigation was carried out mainly to establish suitable methods of purification and to determine the direction of subsequent analysis of tumor extracts, no special care was taken to secure preparations in their purest form. Were these observations to be applied to

TABLE I
Effect of Dialysis on the Total Solids in Chicken Tumor Extract

	Extracts from desiccate				Fresh tissue extracts								Average	
Experiment No.	I		II		III		IIIa		IV		V			
Extract														
	mg. per cc.	per cent	mg. per cc.	per cent	mg. per cc.	per cent	mg. per cc.	per cent	mg. per cc.	per cent	mg. per cc.	per cent	mg. per cc.	per cent
E	7.60		8.65		5.54		5.17		4.70		5.21		6.14	
Sa	1.90	100.0	1.60	100.0	1.50	100.0	1.51	100.0	1.38	100.0	1.52	100.0	1.60	100.0
SaD ₁	0.55	28.9	0.22	12.2	0.56	37.3	0.52	34.4			0.68	44.7	0.52	32.5
SaD ₂	0.60	31.5	0.20	11.1	0.34	22.6	0.42	27.8	0.64	46.3	0.54	35.5	0.45	28.1
SaD ₃	0.60	31.5	0.16	8.8	0.33	22.0	0.39	25.8	0.61	44.1	0.50	32.9	0.43	26.8

The following abbreviations are used throughout the tables: E = tumor extract; Sa = supernatant fluid after adsorption on aluminum hydroxide; SaD₁ = Sa after 1 hour of dialysis; SaD₂ = Sa after 2 hours of dialysis, etc.

In calculating the per cent of dry residue left after dialysis the total solids content of the aluminum supernatant fluid (Sa) has been taken as a basis.

TABLE II
Tumor-Producing Activity of Extracts (Combined Results from Seven Experiments)

Extract	Total No. of inoculations	Average size of tumors*	Positive inoculations per cent	Index of tumor- producing activity
		cm.		
E	27	2.7 x 2.3	100	100
Sa	47	2.6 x 2.0	100	84
SaD ₁	42	1.9 x 1.5	70	46
SaD ₂	39	1.5 x 1.1	55	26
SaD ₃	32	1.2 x 0.9	26	17

* Measurements of tumors were made 14 days after injection.

the purification of extracts on a larger scale much lower figures would be expected.

As shown by the average measurements reproduced in Table II, there was a rapid loss of tumor-producing activity during the process

of dialysis. In the course of the 1st hour this loss of activity amounted to 46 per cent of the tumor-producing activity of the untreated control Sa. After 3 hours treatment, the dialyzed extract retained but 20 per cent of activity of the untreated control.

Before any suggestion could be made regarding the chemical constitution of the extracts, the fate of the active principle during dialysis had to be ascertained. Dialysis had but a slight effect on the reaction of the treated solution. Dialyzed extracts were usually more acid but the change rarely went beyond pH 6.4. Progressive inactivation was first thought to be due to an interphase phenomenon as a result of the shaking. Since the first experiments were conducted at room temperature another explanation concerned the effect of heat and shaking. It was also supposed that the removal of electrolytes from the fluid might have played a part in the inactivation. Each of these explanations was tested. According to our experience the possibility of the agent passing through the membrane was the least probable.

Effect of Shaking upon the Tumor-Inducing Activity at Low Temperature.—The preservation of the activity in extracts over the relatively short period of experimentation appeared to be dependent mainly on the temperature at which the experiment was conducted.

In these experiments the technique devised for dialysis was used, except that glass tubes or glass tubes lined with collodion were substituted for the semi-permeable collodion bags. After periods of 2 or 3 hours shaking, the activity of these solutions was compared with that of the untreated extracts.

On standing 3 hours at room temperature (22°C.) the power to induce tumors exhibited by the freshly prepared supernatants was reduced about 50 per cent, whereas on storage for 3 hours at 12–14°C. there was no reduction in tumor-producing activity. Shaking was found to accelerate inactivation slightly.

Increase of Activity by Dialysis at 12°C.—When dialysis was performed at relatively low temperatures for 3 hours, the material retained by the collodion bags exhibited a tumor-producing activity greater than that of undialyzed extracts kept under the same conditions. These results are illustrated in Tables III and V. This increase of potency was especially marked with extracts submitted to dialysis for periods of 1 or 2 hours; the area of the resulting tumor

sometimes reached twice the size of those produced by the non-dialyzed material. When the treatment was continued over comparatively long periods, from 3 to 5 hours, some inactivation occurred

TABLE III

Effect of Shaking and Dialysis on the Tumor-Producing Activity of Aluminum Supernatant

Experiment No.	Extract	Temperature	Time	Index of tumor-producing activity	
				Shaking plus dialysis	Shaking alone
I	Sa (untreated control)	14	1½	100	100
	Sa (treated)	14	1½	134	100
	Sa (treated)	14	3	96	84
II	Sa (untreated control)	12	3	100	100
	Sa (treated)	12	3	119	98

TABLE IV

*Effect of Salt Concentration on the Activity of Dialyzed Tumor Extracts**

Tumor extract	Temperature	Time	Dry weight	No. of areas injected	Positive inoculations	Average size of tumors	Index of tumor-producing activity
	°C.	hrs.	mg. per cc.		per cent	cm.	
SaD ₁ , dialyzed against distilled water	12	1½	0.42	4	100	2.2 x 1.6	100
SaR ₁ , dialyzed against Ringer's solution	12	1½	9.70	4	100	2.7 x 1.8	136
SaD ₂ , dialyzed against distilled water	12	3	0.29	4	100	1.9 x 1.3	100
SaR ₂ , dialyzed against Ringer's solution	12	3	9.90	4	100	2.2 x 1.6	142
SaD ₃ , alone	12	3	0.29	6	100	2.2 x 1.5	100
SaD ₃ , plus 9 mg. NaCl per cc.	12	3	0.29	6	100	2.1 x 1.6	100

* Measurements of tumors were made 14 days after injection.

even at these temperatures. The increase of tumor-producing activity during the first period of dialysis may result from the diffusion of inhibiting substance present in the tumor extract.

Salt Concentration and Tumor-Producing Activity.—The dry weight

of the dialyzed extract was often reduced as much as 0.2 mg. total solids per cc. This represents a salt concentration inferior to that of isotonic solutions.

Experiments were conducted substituting Ringer's solution for distilled water as the fluid flowing through the glass chamber containing the collodion bag. In another test, 9 mg. per cc. of crystalline sodium chloride were added to the dialyzed portion prior to injection. In this case no difference could be detected in the tumor-producing activity before and after addition of sodium chloride. On the other hand, the data given in Table IV show that the use of Ringer's solution resulted in an increase of the tumor-producing activity.

TABLE V

Effect of Adsorption and Dialysis at 14°C. on the Content of Solids and Potency of Chicken Tumor Extracts

Extract	Time of dialysis	Temperature	Total solids content		Inoculation tests (14 day tumors)				Tumor-inducing activity per mg. dry weight (C) $C = \frac{B}{A}$
			Dry weight (A)	Reduction of dry weight	No. of areas injected	Positive inoculations	Average size of tumors	Index of activity (B)	
	hrs.	°C.	mg. per cc.	per cent		per cent	cm.		
E		14	5.50	—	8	100	2.0 x 1.0	100	18
Sa		14	1.28	78.8	8	100	1.7 x 1.2	102	80
SaD ₁	1½	14	0.42	92.4	8	100	2.3 x 1.7	195	464
SaD ₃	3	14	0.29	94.8	20	100	1.9 x 1.4	133	459*

* Concentration of tumor-inducing activity attained was $459/18 = 25.5$ times that of the extract.

The Effect of Adsorption and Dialysis upon the Concentration of the Agent of Chicken Tumor I.—When water extracts of tumor pulp were fractionated by adsorption and the aluminum supernatant was dialyzed rapidly at 14°C. the tumor activity in the purified product was found to be increased per unit volume of fluid and per unit weight of solid. The total content of solid was reduced from 5.50 mg. per cc. to 0.29 mg. per cc., a reduction of 95 per cent, whereas the tumor-producing activity was increased 33 per cent. It follows that the eventual concentration had 25 times the tumor-producing power of the initial watery extract. These results are given in Table V. The

successive steps in the isolation of the chicken tumor agent are illustrated in Text-fig. 1 where the relative tumor-producing power, total nitrogen content and solids of each fraction are compared.

Effect of Adsorption and of Dialysis upon the Chemical Composition of Chicken Tumor I Extracts

Color Reactions and Tumor-Producing Activity.—In a previous work it was shown that the usual color tests for protein become negative after treatment with aluminum hydroxide (1). However, any protein left in the aluminum supernatant before dialysis was not affected by the treatment, and the biuret test was still positive. The complex carbohydrate which can be removed as a precipitate with gelatin was retained by the collodion bag as shown by positive naphthoresorcin tests. Substances detected by positive ninhydrin tests and by the diazo reaction which were unaffected by adsorption were rapidly eliminated by dialysis. These substances are highly diffusible and the tests became negative after the 1st hour of dialysis. At present there is no color test which could be associated with the tumor-producing activity.

Effect of Dialysis on the Total Nitrogen.—Adsorption removed most of the tumor proteins as determined by color tests and by reduction in nitrogen content. Most of the nitrogen left in the active fraction after adsorption was found to be diffusible. As much as 87 per cent of the nitrogen present in aluminum supernatant prepared from tumor desiccates was discarded by 2 to 3 hours dialysis. Adsorption and dialysis separated 98.6 per cent of the total nitrogen from the fraction carrying the tumor-producing activity. The dialyzed fraction containing the tumor principle had a nitrogen content of 0.01 to 0.05 mg. per cc., or 6.6 to 9.5 per cent of its total solids. The data presented in Table VI are averages of six experiments.

Furthermore, over 20 per cent of the last active fraction was represented by a carbohydrate shown to be independent of the tumor-producing activity. The amount of reducing substances calculated in milligrams of glucose may be used to compute the quantity of carbohydrate present in the active residue, using the formula given by Levene and La Forge for chondroitin-sulfuric acid (10).

Another 25 per cent of the dialyzed fraction was identified as phospholipids by extracting the dry residue with organic solvents.

Then the amount of nitrogen corresponding to the computed amount of carbohydrate and of the phosphatides may be subtracted from the

TABLE VI

*Effect of Adsorption and Dialysis on the Chemical Content of Active Tumor Extracts
Analysis on Extracts from Desiccates and Fresh Tissue Extracts (Combined
Results from Seven Experiments)*

Extract	Total nitrogen			Reducing substances			Total sulfur				Total phosphorus		
	Mg. per cc.	Per cent in dry weight	Relative amount in extracts	Mg. per cc.	Per cent in dry weight	Relative amount in extracts	Mg. per cc.	Per cent in dry weight	Relative amount in extracts	Precipitation with barium	Mg. per cc.	Per cent in dry weight	Relative amount in extracts
E	0.586	9.0	100.0	0.525	8.2	100.0	0.065	1.1	100.0	—	0.056	0.87	100.0
Sa	0.104	6.3	17.7	0.144	8.8	27.4	0.067	4.3		++++	0.006	0.42	10.7
SaD ₁	0.043	5.2	7.3	0.093	19.0	17.7				++	0.006	1.23	10.7
SaD ₂	0.038	7.2	6.4	0.082	18.0	15.6	0.011	2.5		±	0.005	1.24	9.0
SaD ₃	0.036	7.4	6.1	0.080	18.7	15.2	0.011	2.2	16.8	—	0.005	1.24	9.0

The results of the analysis obtained in milligrams per cubic centimeter are also given in per cent of the corresponding total dry weight of each solution. In the last column of each section the relative amount in each fraction has been calculated on the basis of the content of the crude extract taken as 100.

TABLE VII

Proportion of Nitrogen in Unidentified Substances of the Non-Diffusible Fraction

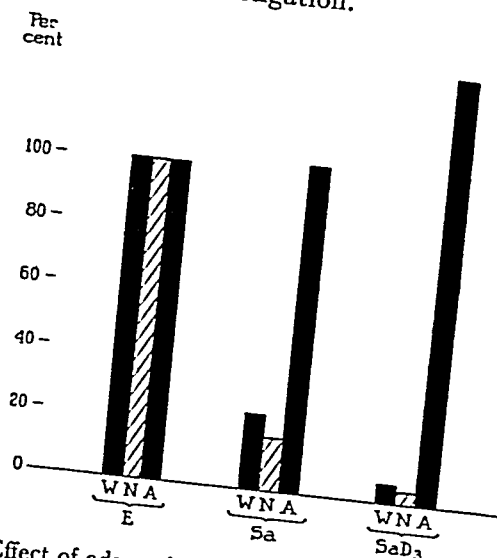
Substances of non-diffusible fraction	Dry weight	Nitrogen for computation	Nitrogen content (determined)	Nitrogen content (computed)	Proportion of nitrogen to dry weight
	mg. per cc.	per cent	mg. per cc.	mg. per cc.	per cent
Total.....	0.430		0.032		7.5
Identified { Carbohydrate.....	0.094	3.0		0.003	
{ Phospholipids.....	0.107	1.7		0.002	
Unidentified.....	0.230			0.027	11.7

Dry weight of carbohydrate was computed from 0.080 mg. glucose by multiplying by $\frac{100}{85.1}$, where 85.1 is the per cent of glucose in chondroitin-sulfuric acid.

Dry weight of phospholipids represents the dry weight of the acetone and alcohol-ether extracts of dried non-diffusible fraction.

total nitrogen found in the last extract. The results of these calculations are shown in Table VII.

Table VII reveals that the unidentified portion carrying practically all the tumor-producing activity of the crude extract had a residual dry weight of 0.23 mg. per cc., of which 11.7 per cent was nitrogen. This percentage of nitrogen of unknown source suggests the presence of a protein as constituent of the purified fraction. The possibility of the protein nature of the tumor principle cannot be excluded and must be tested by further investigation.



TEXT-FIG. 1. Effect of adsorption and of dialysis on the composition and the tumor-producing activity of chicken tumor extracts. The shift in relative values of the total dry solids, nitrogen content, and tumor-producing activity of the aluminum supernatant fluid (Sa) and of the dialyzed fraction (SaD₃) is shown. These values are represented in per cent of the content of the water extract (E), which is taken as 100.

W = total dry solids; N = nitrogen content; A = tumor-producing activity. It will be seen that the total dry weight and nitrogen content were greatly reduced, whereas the tumor-producing activity underwent some increase.

The Concentration of Reducing Substances in Tumor Extracts.—Water extracts of tumor pulp or of tumor desiccates have been found to contain a carbohydrate which can be hydrolyzed by acid to yield about 0.525 mg. per cc. of reducing substances, calculated as glucose. This carbohydrate was identified as the prosthetic group of a mucoprotein found in abundance in all Chicken Tumor I preparations.

Usually over 70 per cent of this compound in terms of glucose was eliminated by adsorption to alumina gel. In spite of this adsorption the ratio of reducing substances to the total solids was practically the same in the aluminum supernatant fluid as in the water extract; e.g., 8.8 and 8.2 per cent respectively. Less than half of the reducing substances which were not adsorbed were readily diffusible and this amount diffused during the 1st hour of dialysis. Since greater proportions of other components were separated by dialysis, the ratio of reducing substances to total solids increased in the dialyzed material. After 3 hours dialysis the proportion of reducing substances was 16 per cent in purified extracts prepared from tumor pulp and 21.8 per cent in purified extracts prepared from tumor desiccates. The results are presented in Table VI and show that about 15 per cent of the reducing substances is retained by the dialyzed aluminum supernatant. If these values are calculated in terms of chondroitin-sulfuric acid, more than 22 per cent of the non-diffusible fraction is shown to be represented by this inactive product. The activity remains with the other unidentified constituents.

Total Sulfur.—No free sulfate could be detected in Berkefeld filtrates or in pulp extracts of Chicken Tumor I by the usual methods of precipitation. Some sulfate from the alumina gel which was used to adsorb inactive substances was released during adsorption (1). Therefore, determination of the total sulfur in aluminum supernatant fluid could not be accomplished without preliminary treatment with barium. The use of dialysis eliminated the need of preliminary barium precipitation.¹ The extraneous sulfate in aluminum supernatant fluid was separated by diffusion after the 3rd hour, as shown by precipitation tests with barium.

As shown in Table VI, total sulfur in the water extract amounted to about 0.065 mg. per cc., or 1.1 per cent of the total dry solids. The total sulfur in dialyzed fractions amounted to as high as 0.011 mg. per cc., or 2.2 per cent of the dry weight of the purified fraction. Ac-

¹ Precipitation of inorganic sulfate, induced in active tumor extracts by means of barium salts, had no harmful effect on the tumor-producing activity of the extract provided the precipitation was carried out at a convenient pH. Either barium hydroxide or barium acetate solutions adjusted to pH 7.0 by means of acetic acid were used for this purpose.

cordingly, 16.8 per cent of the total sulfur present in the original water extract was retained after adsorption and dialysis. No attempts have been made to determine the amounts of neutral sulfur or ethereal sulfate or to establish what part of the non-diffusible sulfur should be ascribed to the tumor carbohydrate. The sodium nitroprusside reaction for sulphydryl groups was always negative.

Total Phosphorus.—The results of analysis of the tumor material showed that the total phosphorus content of the water extract was about 0.056 mg. per cc. or 0.87 per cent. Of this amount at least 90 per cent had been removed by adsorption to aluminum hydroxide. A very small portion of the unadsorbed phosphorus diffused during dialysis and probably represented some of the inorganic phosphate present in the water extract which had failed to combine with the gel. After dialysis the non-diffusible phosphorus amounted to 1.24 per cent of the solids of the dialyzed fraction, or 0.005 mg. per cc. of solution. A considerable part, if not all, of this non-diffusible phosphorus belonged probably to phospholipids which were found to account for about 25 per cent of the solids of the dialyzed extract. The averaged results from seven experiments are given in Table VI. The amount of phosphorus detected in the dialyzed fraction was too small to permit of a discussion of its significance. A definite estimation of the rôle played by phosphorus compounds in the production of tumors must await analyses of larger quantities of material.

Extraction of Lipoids

Lipoid Extraction of Tumor Desiccates.—3 gm. of finely sifted tumor desiccate, prepared in the usual manner, were extracted 3 times with dry acetone. Each extraction consisted in shaking the powder with 120 cc. dry acetone for 3 hours at room temperature. After the last acetone extraction, the acetone-insoluble part of the powder was treated similarly with an alcohol-ether mixture (2 volumes absolute alcohol and 1 volume absolute ether). The acetone and alcohol-ether extracts were evaporated to dryness and the residue was weighed. The tumor material was rid of traces of solvents by evaporation *in vacuo* in the presence of sulfuric acid. The fat-free powder was extracted with water and inoculation tests were made for tumor-producing activity.

The amount of fat-free tumor powder recovered was 2.581 gm. and the total lipid amounted to 0.417 gm.; 14 per cent of the desiccated tumor tissue was found to be soluble in fat solvents. Inoculation

Usually over 70 per cent of this compound in terms of glucose was eliminated by adsorption to alumina gel. In spite of this adsorption the ratio of reducing substances to the total solids was practically the same in the aluminum supernatant fluid as in the water extract; e.g., 8.8 and 8.2 per cent respectively. Less than half of the reducing substances which were not adsorbed were readily diffusible and this amount diffused during the 1st hour of dialysis. Since greater proportions of other components were separated by dialysis, the ratio of reducing substances to total solids increased in the dialyzed material. After 3 hours dialysis the proportion of reducing substances was 16 per cent in purified extracts prepared from tumor pulp and 21.8 per cent in purified extracts prepared from tumor desiccates. The results are presented in Table VI and show that about 15 per cent of the reducing substances is retained by the dialyzed aluminum supernatant. If these values are calculated in terms of chondroitin-sulfuric acid, more than 22 per cent of the non-diffusible fraction is shown to be represented by this inactive product. The activity remains with the other unidentified constituents.

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¹ Precipitation of inorganic sulfate, induced in active tumor extracts by means of barium salts, had no harmful effect on the tumor-producing activity of the extract provided the precipitation was carried out at a convenient pH. Either barium hydroxide or barium acetate solutions adjusted to pH 7.0 by means of acetic acid were used for this purpose.

cordingly, 16.8 per cent of the total sulfur present in the original water extract was retained after adsorption and dialysis. No attempts have been made to determine the amounts of neutral sulfur or ethereal sulfate or to establish what part of the non-diffusible sulfur should be ascribed to the tumor carbohydrate. The sodium nitroprusside reaction for sulfhydryl groups was always negative.

Total Phosphorus.—The results of analysis of the tumor material showed that the total phosphorus content of the water extract was about 0.056 mg. per cc. or 0.87 per cent. Of this amount at least 90 per cent had been removed by adsorption to aluminum hydroxide. A very small portion of the unadsorbed phosphorus diffused during dialysis and probably represented some of the inorganic phosphate present in the water extract which had failed to combine with the gel. After dialysis the non-diffusible phosphorus amounted to 1.24 per cent of the solids of the dialyzed fraction, or 0.005 mg. per cc. of solution. A considerable part, if not all, of this non-diffusible phosphorus belonged probably to phospholipids which were found to account for about 25 per cent of the solids of the dialyzed extract. The averaged results from seven experiments are given in Table VI. The amount of phosphorus detected in the dialyzed fraction was too small to permit of a discussion of its significance. A definite estimation of the rôle played by phosphorus compounds in the production of tumors must await analyses of larger quantities of material.

Extraction of Lipoids

Lipoid Extraction of Tumor Desiccates.—3 gm. of finely sifted tumor desiccate, prepared in the usual manner, were extracted 3 times with dry acetone. Each extraction consisted in shaking the powder with 120 cc. dry acetone for 3 hours at room temperature. After the last acetone extraction, the acetone-insoluble part of the powder was treated similarly with an alcohol-ether mixture (2 volumes absolute alcohol and 1 volume absolute ether). The acetone and alcohol-ether extracts were evaporated to dryness and the residue was weighed. The tumor material was rid of traces of solvents by evaporation *in vacuo* in the presence of sulfuric acid. The fat-free powder was extracted with water and inoculation tests were made for tumor-producing activity.

The amount of fat-free tumor powder recovered was 2.581 gm. and the total lipid amounted to 0.417 gm.; 14 per cent of the desiccated tumor tissue was found to be soluble in fat solvents. Inoculation

tests made before and after treatment of the tumor desiccates showed that the tumor-producing activity was unaffected by acetone and alcohol-ether extraction.

Lipoid Extraction of Tumor Extracts.—The various tumor fractions were distributed in 3 cc. samples in small, tared dishes (pyrex), and evaporated to dryness on the water bath. The residue was a thin, continuous film, adherent to the glass. The drying was completed by heating 2 hours at 105°C. When the extraction was to be followed by inoculation tests for activity the material was dried in the presence of sulfuric acid at a reduced pressure of 10 mm. Hg. A loose web, unfit for quantitative manipulation, forms if the evaporation is per-

TABLE VIII
Combined Lipoid Extraction by Acetone and Alcohol-Ether Mixture

Experiment No.....	VIIa				VIIb				IX				Combined results			
Extract	Total solids	Lipoid extraction			Total solids	Lipoid extraction			Total solids	Lipoid extraction			Total solids	Lipoid extraction		
		Amount extracted	Relative amount			Amount extracted	Relative amount			Amount extracted	Relative amount			Amount extracted	Relative amount	
	mg. per cc.	mg. per cc.	per cent	per cent	mg. per cc.	mg. per cc.	per cent	per cent	mg. per cc.	mg. per cc.	per cent	per cent	mg. per cc.	mg. per cc.	per cent	per cent
E	5.50	1.34	24.3	100.0	5.86	1.35	23.2	100.0	7.45	1.70	22.8	100.0	6.21	1.46	23.4	100.0
Sa	1.26	0.69	54.7	51.4	1.55	0.76	49.0	56.2	1.68	0.85	50.5	50.0	1.49	0.76	51.4	52.0
SaD ₃	0.30	0.08	26.6	5.9	0.80	0.23	28.7	17.0	0.26	0.06	25.3	3.9	0.45	0.12	26.8	8.2

formed at lower pressures. The dry material was covered by 5 cc. of the solvent and the dishes kept in a closed jar for 3 hours. Then the solution was decanted and the residue treated with another sample of solvent. The extraction was performed 3 times with dry acetone and 3 times with an alcohol-ether mixture consisting of 2 volumes of absolute alcohol and 1 volume of ether. The acetone and alcohol-ether extracts were analyzed separately or together. The soluble fractions were recovered by evaporation to dryness on a water bath, and the amount extracted determined by weight of the residue.

23.4 per cent of the water extract treated in this manner was found to be soluble in organic solvents. About one-half of this lipoid fraction was separated by adsorption to aluminum hydroxide gel. Over 50 per cent of the solids in the aluminum supernatant fluid was soluble in organic solvents. Furthermore, it was shown that most of this

presumed lipoid was diffusible and consisted mainly of neutral fat and fatty acids. The non-diffusible lipoid portion represented 26.8 per cent of the active, undialyzable residue and possessed the general characteristics of phosphatides: it was a white, partially crystalline material, containing phosphorus to 2.3 per cent.

DISCUSSION

The present study was undertaken on the assumption that the causative agent, extracted in cell-free preparations from the tumor tissues, may be a soluble constituent of the active extracts. If this hypothesis be correct it should be possible to identify the neoplastic principle by adequate biochemical methods. In the present investigation no attempts were made to isolate the tumor principle by direct chemical means, since the use of chemical methods offers great difficulties owing to the complexity of the tumor extract and to our ignorance concerning the chemical properties of the agent itself. The method adopted comprised the identification of the chief constituents of the tumor extract and their removal without impairment of the tumor-producing activity.

The proteins present in the water extract of chicken tumor tissues were found to consist for the most part of mucoproteins, probably a product of the tumor cells. Another constituent of tumor extracts is a carbohydrate which may be a decomposition product of mucoproteins or a direct product of the same cellular activity.

Through successive elimination of various inactive products the tumor principle has been obtained in a state of relative purity. From typical results it was calculated that the portion of the purified extract not yet chemically identified and carrying the entire activity of the original material had a total dry weight of 0.17 mg. per cc., with a nitrogen content of 11.7 per cent. In this instance the methods of adsorption and dialysis brought the amount of inactive constituents separated during the purification to 97 per cent of the original solids. The relatively high nitrogen content of the last active fraction suggests that protein may be a part of the purified material.

15.2 per cent of the reducing substances and 16.6 per cent of the sulfur existing in the water extract are retained in the dialyzed fraction. This parallelism in quantity may indicate that most of the

sulfur detected in the water extracts of Chicken Tumor I is derived from the tumor carbohydrate. Another important portion, amounting to one-fourth of the active residue, was found to consist of phospholipoids. Further fractionation of the residue and identification of the additional chemical products must be continued in order to determine whether the tumor-producing property may be connected with one of these chemical constituents. Experiments are under way to separate the non-diffusible lipoids from the residual proteins.

At this stage the chemical nature of the tumor agent cannot be discussed. The only test available at present for the detection of the tumor principle is the biological test. In view of the lack of information regarding the amount of specific agent or the number of units necessary for the induction of a positive test, *i.e.* the initiation of a tumor, it is impossible to say whether chemical determinations on the material now available will prove informative. The lack of information concerning the real nature of the filterable agents causing chicken tumors seems to justify our attempt to apply biochemical methods to their study.

Whatever the outcome of the work, the identification of the inactive chemical constituents of chicken tumor extracts and the use of purified solutions should facilitate the study of the properties of the specific agents.

SUMMARY

The fractionation of active chicken tumor extracts has been continued.

1. By a rapid method of dialysis the diffusible fraction of the treated extract, representing about 75 per cent of the solids, has been eliminated without reducing the tumor-producing activity.

2. Combined methods of fractionation have resulted in the elimination of as much as 95 per cent of the total solids of the extract as inactive constituents. Since there was some concomitant enhancement of the activity of the agent the results were equivalent to a 25-fold concentration of it in terms of dry weight.

3. The chemical content of the undiffusible fraction has been determined in terms of total nitrogen, reducing substances, sulfur, phosphorus, and lipoids.

4. The evidence points to a protein and a phospholipoid as the principal constituents of the active residue. Further fractionation is being attempted with a view to connecting the tumor-producing activity with one of its remaining constituents.

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THE DEMONSTRATION OF A TUMOR GROWTH-INHIBITING FACTOR FROM NORMAL HUMAN CONNECTIVE TISSUE

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Murphy and his coworkers among others have shown that an inhibiting factor can be isolated from fowl sarcoma, capable of neutralizing the causative agent of this tumor (1). This factor also prevents the growth of transplantable mouse sarcomas, thus demonstrating that it is not a species-specific agent (2). In a more recent study these investigators have further extended their search for agents which have the power of inhibiting growth in transplantable mammalian carcinomas. They have found that extracts from normal animal embryo skin and placenta yield substances which exert definite retarding action on two transplantable carcinomas of mice, but have no effect on mouse sarcomas (3, 4). No definite inhibition was noted in fresh extracts, desiccation of the material being necessary to produce the optimum inhibitory effect.

As soon as the methods were available, an investigation was undertaken in our laboratories on sources of inhibitors in human tissues. We have studied extracts of fresh human placenta, of pectoral muscle, of rectus sheath, and of uterine fibroids. After some preliminary trials it seemed best to limit our work to one type of tissue extract and to defer experimentation on the other tissues to a later date. Since the extracts of human connective tissue (rectus sheath) have indicated more of promise than the others, we confined our attention to these.

Material and Method

In all experiments white rats approximately 4 months of age were used. These rats were bred from our own stock strain. This strain has been used extensively here in connection with the test tumor and is not resistant to it. The rat tumor,

tumor had attained an average size of 3.0 x 1.5 cm. whereas the fresh human fascia extract had caused a complete inhibition in the case of the others. This identical result was attained in three separate experiments. In three other experiments results as indicated in Fig. 2 were noted. The growth in the control series behaved as usual but

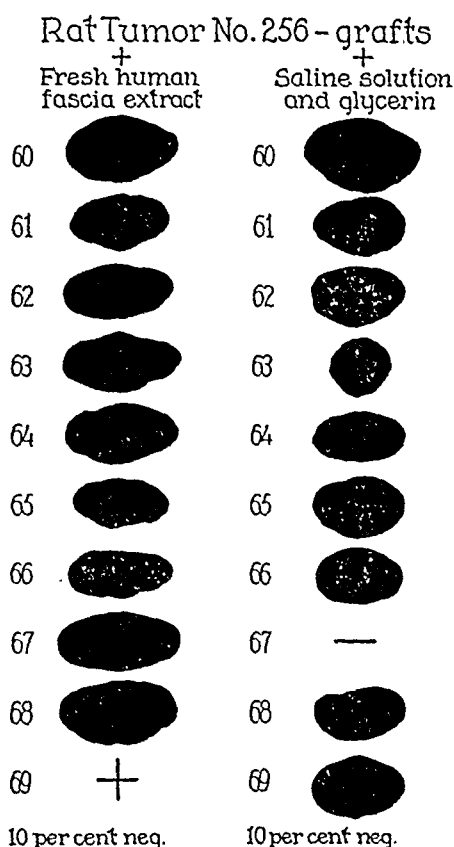


FIG. 3. The connective tissue extract caused decided retardation of the tumors for 3 weeks. After that period the retarded tumors grew faster than the controls. This occurred in one experiment only. The silhouettes are one-third the size of the growths.

there was not complete inhibition of all of the test tumors, though a definite retardation was noted in all cases. In two of these experiments three small tumors grew in the test animals, and in the third experiment five such tumors appeared. In a seventh experiment the controls grew as usual, but although there was a definite slowing and

TABLE I

Material inoculated with Rat Tumor 256	Experiment No.	No. of test rats inoculated	No. of test tumors at 5 wks.	Average size at 5 wks.	No. of controls inoculated	No. of tumors at 5 wks.	Average size at 5 wks.
				<i>cm.</i>			<i>cm.</i>
Fresh human rectus sheath	1	10	0	—	10	9	3.13 x 1.75
	2	10	0	—	10	9	3.20 x 1.54
	3	10	5	1.2 x 0.42	10	10	3.16 x 2.01
	4	10	3	0.83 x 0.46	10	10	3.18 x 1.64
	5	10	0	—	10	9	3.04 x 1.72
	6	10	3	0.92 x 0.43	10	8	3.15 x 1.96
	7	15	14	3.75 x 1.95	15	14	3.05 x 2.02
Totals.....		75	25		75	69	

Rat Tumor No. 256 - grafts

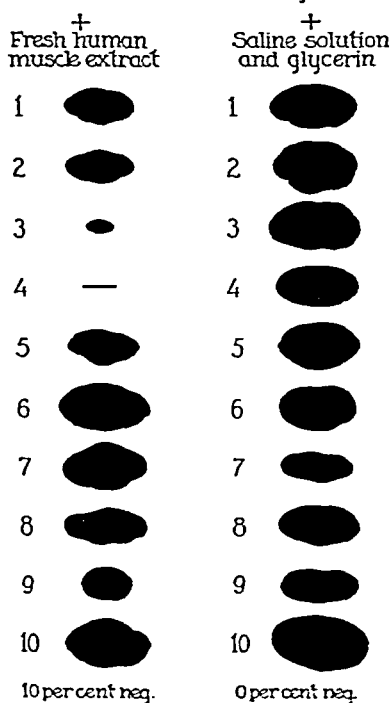


FIG. 4. Muscle tissue extract had practically no effect. The silhouettes are one-third the size of the growths.

inhibition to the 3rd week, 14 of the 15 test tumors not only grew but actually seemed to grow faster than the controls when once the inhibition had been overcome. At the end of the 5 weeks period these test tumors measured slightly more than the controls. This is shown in Fig. 3. The results of all these experiments are listed in Table I.

In contrast to the action of extracts of connective tissue we have presented in Fig. 4 the outcome of a typical experiment with extracts of human muscle, in which exactly the same method of preparation was utilized. Here any effect noted was well within the normal variation in behavior of the tumor.

DISCUSSION

These observations are perhaps too few to warrant any sweeping deductions. It is fully realized that much more evidence must be accumulated with other test tumors. This is now being done in our laboratories. The results noted, however, may be taken to indicate trends which can be proved or disproved later. The work of Murphy and his collaborators seems to point to the interaction of two forces inherent in every cell to maintain it in a balanced state. Under normal conditions the balance is perfectly controlled. These regulators in each cell must be small in quantity and easily changed over to some other form. The experiments made by Murphy and his coworkers show that it is possible to extract both stimulators and inhibitors from the tissues. It has seemed to us from long observations on healing wounds that there must be a mutual interplay of forces between tissues derived from different embryological layers. Thus, in every healing wound the balance must be carefully adjusted between the epithelium on the one side and the granulation tissue on the other. It seems probable that embryonic types of connective tissue contain inhibitors for epithelium at certain stages, *i.e.* near the scar-forming period; and that earlier in the red active stage the epithelium is stimulated by the granulation tissue. It was for this reason that we decided to try extracts of the more undifferentiated type of connective tissue against an epithelial tumor. Rat Tumor 256 has been described as a pure carcinoma which "takes" in almost 100 per cent of transplants, and hence it was selected as the test object. Perhaps this selection has been unfortunate since it has been

found on further investigation that not all authorities regard Rat Tumor 256 as a pure carcinoma. By some it is held to be a carcinosarcoma. It has also been observed to grow in only 75 per cent of transplants in some laboratories. The experience of several workers in this University has been that it grows in about 90 per cent of transplants. There have been periods of growth fluctuation when it has receded, but the growth of the control grafts indicates that these experiments were not conducted at such a period. The extracts of connective tissue were not desiccated in our observations, another difference from Murphy's experiments. In future experiments, this will be done as it apparently enhances the potency of the inhibiting factor. Extracts of muscle—a highly differentiated form of connective tissue—were not effective against the growth of transplants of Rat Tumor 256.

Objection may be raised to these experiments in that the inhibitor substance came from a heterologous source. In answer to this, there was not observed any toxic reaction or condition which might be regarded as allergic. Furthermore, the failure of human muscle extracts prepared in the same way to influence the tumor growth serves as adequate control to this point. Homologous rat fascia is so thin that it does not lend itself to trial. In Murphy and Sturm's most recent papers the inhibiting effects from some heterologous tissues were quite definite.

A few attempts to cause recession of established transplants of Rat Tumor 256 by intraperitoneal and subcutaneous inoculations were not successful. This phase of the problem must be deferred for the present.

SUMMARY

Extracts from fresh normal human connective tissue (rectus sheath) exhibited a decided inhibiting action on grafts of Rat Tumor 256 transplanted into test animals. There was complete inhibition of tumor growth in 66 per cent of the animals; there was a marked retardation in the rate of growth in another 15 per cent, the tumors in these instances being much smaller than the controls transplanted at the same time. In only one experiment was there failure to obtain a lasting growth-inhibitory effect. The tumors in these animals (19

per cent) although showing an initial retardation in growth, apparently overcame the restraint and at the end of 5 weeks were larger than the control series. Extracts from fresh normal human muscle tissue, on the other hand, showed no such inhibiting action on grafts of Rat Tumor 256.

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PRESSOR SUBSTANCES FROM THE BODY FLUIDS OF MAN IN HEALTH AND DISEASE*

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It has been a theory of long standing that high blood pressure is due to circulation of a pressor substance acting on the peripheral blood vessels. Convincing proof either for or against this conception does not appear to have been established. It seemed to us, therefore, that the problem was worthy of intensive study.

Much has been written on the subject of pressor substances in blood, but due to the fact that the methods employed in the preparation and testing of the extracts are so divergent, only certain of them need be considered here.

Vasoconstrictor substances in the alcoholic extract of blood from hypertensive patients have perhaps been demonstrated (1, 3, 9, 10), but their presence has been denied (11-13).

Bohn (9, 10) prepared alcoholic extracts of blood and tested them on curarized cats, anesthetized with ethyl urethane. He concluded that a pressor substance was present in the blood of patients suffering from nephritic hypertension, from eclampsia, and from malignant nephrosclerosis, but, without exception, blood from patients suffering from essential hypertension or normal subjects contained none. The substance was found to be ultrafiltrable and rendered inactive by exposure to ultraviolet irradiation or to alkali. It also acted as an antidiuretic. Bohn believes that the material is pituitrin. These experiments were taken as proving Volhard's hypothesis that the mechanisms of nephritic and of essential hypertension are fundamentally different, the former depending on the presence of a chemical substance and the latter being predominantly of a nervous origin.

Marx and Hefke (14) also found that alcoholic extracts of blood from nephritic hypertensive patients induced a prolonged rise of blood pressure level in non-anesthetized dogs. Ultrafiltrates were not active, contrary to Bohn's results. Extracts from the blood of normal persons were inactive, while those from epileptic patients, shortly before or during convulsions, were strongly positive. No relationship between the height of the pressure and the amount of pressor substance was found.

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Plasma of eclamptic patients acidified (pH 4 to 4.5) with acetic acid and passed through an ultrafilter was studied by Anselmino and Hoffmann (15-17) in anesthetized rabbits. They appeared able to demonstrate the presence of a substance in the blood of eclamptic patients and those suffering from toxemia of pregnancy that they considered identical with the pressor hormone of the posterior pituitary body. They obtained active antidiuretic ultrafiltrates only when edema was present in the patient, and active pressor extracts only when the blood pressure was over 180 mm. of mercury. The amount of the substance in the blood appeared to parallel the severity of the symptoms. Byrom and Wilson (18) have failed to find antidiuretic hormone in ultrafiltrates of plasma of ten cases of pre-eclamptic toxemia and three cases of eclampsia with edema.

Many investigators have presented evidence which suggests that the pressor hormone of the posterior pituitary body may be in part responsible for the maintenance of vascular tone (20-33). Others have denied that the evidence is convincing (34-38). Hoyle (37) has clearly demonstrated that normal spinal fluid contains, at most, only a trace of pituitrin, nor is it increased in fluid from patients suffering from nephritis or essential hypertension.

Efforts to detect adrenalin or adrenalin-like substances in the blood by the use of the intestinal segment method or the Trendelenburg frog preparation have led to contradictory results (39-51). No proof has yet been offered that the blood *in vivo* contains such substances in active form.

Chemical Qualities of Pressor Extracts

Extraction and Concentration.—Blood was drawn into a syringe containing heparin from the antecubital vein of the patient. Moderate compression of the arm by the use of a tourniquet did not affect the amount of pressor substance. Both sodium citrate and potassium oxalate have been employed as anticoagulants and, while it could not be definitely proven that they interfered with the animal testing, heparin seemed less likely to complicate the pharmacological assay.

The blood was immediately centrifuged (3 minutes) and the plasma pipetted into 95 per cent ethyl alcohol in the proportion of 10 cc. plasma to 90 cc. alcohol. 10 minutes were usually required for the whole procedure. The flasks were then placed in the ice box for from 2 to 24 hours, the precipitated protein and lipid filtered off, employing strong suction, and the clear filtrate concentrated to one-half the original plasma volume. The removal of the alcohol was carried out in a Claissen flask with water-cooled condenser under vacuum (1 to 2 mm.). The temperature in the flask did not rise above 20°C.

It is essential that all alcohol be evaporated from the extract. Small amounts allowed to remain in solution markedly accentuate the action of the pressor substance. It seemed desirable as a control whenever a great rise had been observed in the level of the blood pressure of the test animal to evaporate the extract still further under vacuum. The type of blood pressure curve resulting from injection of extract containing alcohol is similar to that from alcohol-free extract except

that the level to which the pressure rises is much greater. This precaution must be rigidly observed.

An alternative method was also employed, in which, after removal of the alcohol, absolute alcohol was added in the proportion of 40 cc. for every 1 cc. of concentrate. The mixture was quickly chilled to 4°C ., and after standing 6 hours filtered, and the alcohol removed under vacuum.

Other solvents than alcohol have been tried. Acetone extracts were in almost every case either moderately or strongly depressor in action. Ether extracts were indifferent, being neither pressor nor depressor.

Extracts prepared by the ordinary Folin-Wu phosphotungstic acid precipitation were inactive. Trichloroacetic acid dissolved more depressor than pressor substance and, furthermore, the sodium acetate formed on adjusting the pH to that of the body interfered with the animal testing.

Precipitation by tannic acid at pH 3.0 proved an effective protein precipitant, but the removal of the excess tannic acid by the addition of barium hydroxide was troublesome. Excess barium was removed with sulfuric acid. While a small amount of residual tannic acid does not interfere with animal testing, any residual barium may seriously disturb the results. The pressor substance does not appear to be destroyed by the treatment.

Shifting the hydrogen ion concentration of plasma to pH 4.5 by the addition of normal acetic acid had no effect on the efficiency of the alcohol extraction. The concentration of the alcohol employed in the extraction, as long as the protein precipitation was complete, also affected the results insignificantly.

The extract prepared by the alcohol method was usually protein-free as measured by the sulfosalicylic acid method. Often relatively large amounts of lipids were present, and while it was demonstrated that they did not interfere in the animal testing, the lipids were usually removed by cooling the extract to 8°C . and decanting the clear fluid. The extracts were tested usually within 12 to 24 hours, but often immediately after concentration. Serial experiments showed that the time of extraction with alcohol in the refrigerator made little difference in the amount of pressor material extracted. After removal of the alcohol, however, the extracts, especially if kept at room temperature, tended to lose their pressor quality and become progressively more depressor. If extracts were not tested immediately, they were kept at 8°C . It has been our experience that the technique for the preparation of extracts must be carried out with exactness and rapidity, otherwise depressor substances are liberated. These substances may be fatal to the test animal unless caution is used.

Coagulation as a Factor in the Production of Pressor Substance.—

Every care was taken to prevent coagulation by the use of the anti-coagulant heparin, but the necessary manipulations during extraction might have allowed some alteration to occur. After it had been shown that ascitic fluid also contained pressor substance a way was open for excluding coagulation as a factor in the production of the blood pressure-elevating principle. During a paracentesis ascitic fluid was allowed to flow directly from the trocar into alcohol. The extract of this fluid was neither more nor less active than heparinized or citrated ascitic fluid. It seems reasonable therefore to suppose that the substance is present in plasma and is not produced as the result of coagulation.

Ultrafiltration.—After exhaustive investigation it became evident that ultrafiltration could not be utilized for the separation of pressor substance from plasma.

Various methods for the preparation of ultrafiltrates have been employed. Collodion sacs of various porosity (100 cc. capacity), and pear-shaped porcelain thimbles on which acetic acid collodion (6 to 8 per cent Shering-Kalbaum¹) was deposited as a membrane, were found convenient. Ultrafiltrates were also prepared by high pressure (150 kilos per sq. cm. of nitrogen) filtration² through membranes of various density (30 to 400 minutes). The high pressure apparatus offered the great advantage that 10 cc. or more of ultrafiltrate could be obtained in $\frac{1}{2}$ hour, the time depending on the porosity of the filter membrane.

Ultrafiltrates were prepared from plasma at normal pH and from plasma which had been made acid (pH 4.5) by the addition of acetic acid. Neutralization of the acid dialysates (to phenolphthalein) was performed immediately before injecting into the animal. Ordinarily the ultrafiltrate was not concentrated. When concentration was desired it was effected below 20°C. with the aid of a vacuum. The ultrafiltrates were clear and protein-free as estimated with the sulfosalicylic acid reagent. 52 ultrafiltrates have been tested.

Partition.—Active extracts prepared by the alcohol method were extracted with chloroform in a separatory funnel. The solvent was removed under vacuum after the addition of water. Such aqueous extracts were vaso-active. Many experiments have shown that the pressor substance is extracted from aqueous solution by this strong organic solvent, often almost completely. Reversing the extraction,

¹ The preparation of these ultrafiltrates was undertaken with the help of Dr. K. J. Anselmino of Düsseldorf.

² Pfaltz and Bauer apparatus.

that is extracting the substance from chloroform after acidification, was not so successful, only about 20 per cent being so recoverable. Ethyl acetate also removes some of the pressor substance from the water phase but does not appear as efficient as chloroform. Plasma which has been allowed to stand at room temperature yields an extract which is powerfully depressor and partition of this extract with chloroform does not leave the depressor substances in the water phase.

Heat Stability.—Plasma extract may be heated to boiling for about 1 minute with but partial loss of pressor activity (nine experiments). Longer heating even at lower temperatures either liberates depressor substances in such quantity that they overshadow the pressor principle or else the pressor substance is destroyed leaving the depressor substances unantagonized. The experimental evidence does not allow us to decide which reaction had occurred.

Is the Pressor Substance Lipid in Nature?—The gross fats may be frozen out of the extracts without substantial loss of pressor activity. These fats in aqueous emulsion when injected were not vaso-active. Further removal of phosphatide was achieved by precipitation with a large excess of acetone and allowing the precipitate to agglomerate at a temperature just above freezing. The supernatant liquor after the addition of water and the removal of the acetone under vacuum remained vaso-active. It thus seems unlikely that any of the ordinary lipids are involved in the activity of these extracts.

Vascular Responses of the Test Animals

Method.—Cats weighing 3 to 4 kilos which had been without food for 18 hours were usually employed as test animals. Lactating or pregnant animals responded to vascular stimuli in a peculiarly irregular manner, hence were not used in these experiments. The animal was anesthetized and a tracheal cannula inserted. Both vagi were then cut and the blood pressure recorded, by a mercury manometer, either from the right carotid artery, cannulated well below the cricoid cartilage, or from the femoral artery. Interference with the circulation of the thyroid gland was avoided. The femoral artery was employed for blood pressure measurement when injury to the carotid sinus and aortic nerves was to be avoided. Sodium citrate, 20 per cent, was employed as anticoagulant in the manometer tubing because it has been found that should some of the solution flow into the vascular system its action can be quickly nullified by the intravenous injection of 5 per cent calcium lactate. The injections of warmed extract, usually of 5 cc. volume, were made slowly and evenly into a cannulated femoral vein. The ani-

imals were kept warm and the temperature noted from time to time by means of a rectal thermometer.

Anesthetics.—A variety of anesthetics have been studied with regard to their suitability for pressor extract assay. Ether proved satisfactory when administered in gentle puffs of warm air from a windshield wiper type of artificial respirator but with the tracheal cannula open to the outside air. It was found essential to keep the respiration normal and unimpeded by the respirator.

Ethyl urethane given subcutaneously 3 to 4 hours before the experiment in doses of 7 to 10 cc. of a 25 per cent solution was found equally satisfactory and was employed in many of our experiments.

Amytal (iso-amyl ethyl barbituric acid) administered in doses of 60 mg. per kilo produced a smooth anesthesia without marked fluctuations in the level of the blood pressure but the response to extract was not as great as that when ether or ethyl urethane was employed. This lack of sensitivity is surmised to be due to the fact that this barbituric acid derivative stabilizes the autonomic nervous system in such a manner that vasomotor responses are buffered.

Pento-barbital (1-methyl-butyl ethyl barbituric acid) administered intraperitoneally in doses of 40 mg. per kilo gave an excellent anesthesia and the responses were similar to those following amytal narcosis, with the exception that early in the course of the experiment, depressor responses were likely to be elicited by extracts of known pressor activity.

Chloretone (0.4 gm. per kilo given by stomach tube) depressed the responsiveness to pressor extract to a marked degree.

Course of the Vascular Responses to Pressor Substance.—It was observed that the same extract at one time produced a marked rise in the animals' blood pressure, whereas, later in the course of the experiment either the response was small or none was elicited. That the extract had deteriorated in so short a time did not seem probable. Extracts which had assayed both active and inactive tested on the same animal were reassayed the next and subsequent days on other animals. Again the same variability was noted. The conclusion seemed justified that the variability lay in the response of the animal and not in the extracts.

On analyzing the data derived from the blood pressure records of a large number of cats it became evident that under the conditions of our experiments, especially when ether was employed as anesthetic, fairly definite periods could be recognized during which the magnitude of the response was reasonably constant. Since the responsiveness during the different periods varied greatly it became evident that to obtain comparable results the assay would have to be performed dur-

ing the same period. This observation was established on such a large number of animals, because the truth or falsity of this observation lies at the crux of the important problem of the quantitation of the pressor substance.

During Period 1 (Fig. 1) the blood pressure is high and tends to fall rather rapidly and progressively. The animal is usually but slightly responsive to plasma extract during this period, indeed pressor extracts may produce depressor reactions. Period 2 is characterized by a moderately low (about 108 to 120 mm. Hg) steady blood pressure and may last for 1 to 3 hours. It is during this interval that the animal is most sensitive to pressor substances. As the third period ap-

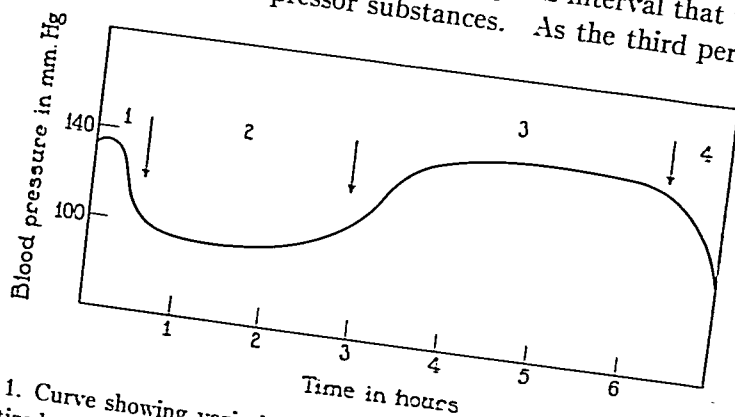


FIG. 1. Curve showing variations in blood pressure exhibited by a cat while anesthetized with ether.

proaches the pressure very gradually rises to a level usually greater than the initial pressure. At this level it remains almost fixed and will carry on for hours without significant alteration. The responses are exceedingly sluggish. It is during this period that an extract which had previously proven active may seem entirely devoid of pressor activity. Period 4 sets in when the animal is fast failing. During this period the responses are usually very irregular and are often confused by asphyxial blood pressure waves. Experiments performed to determine whether this course of events is the normal one, under prolonged anesthesia indicate that it is (Fig. 2). It must be recognized that this division of the vascular reactivity into periods is arbitrary. The periods are not so distinct when the

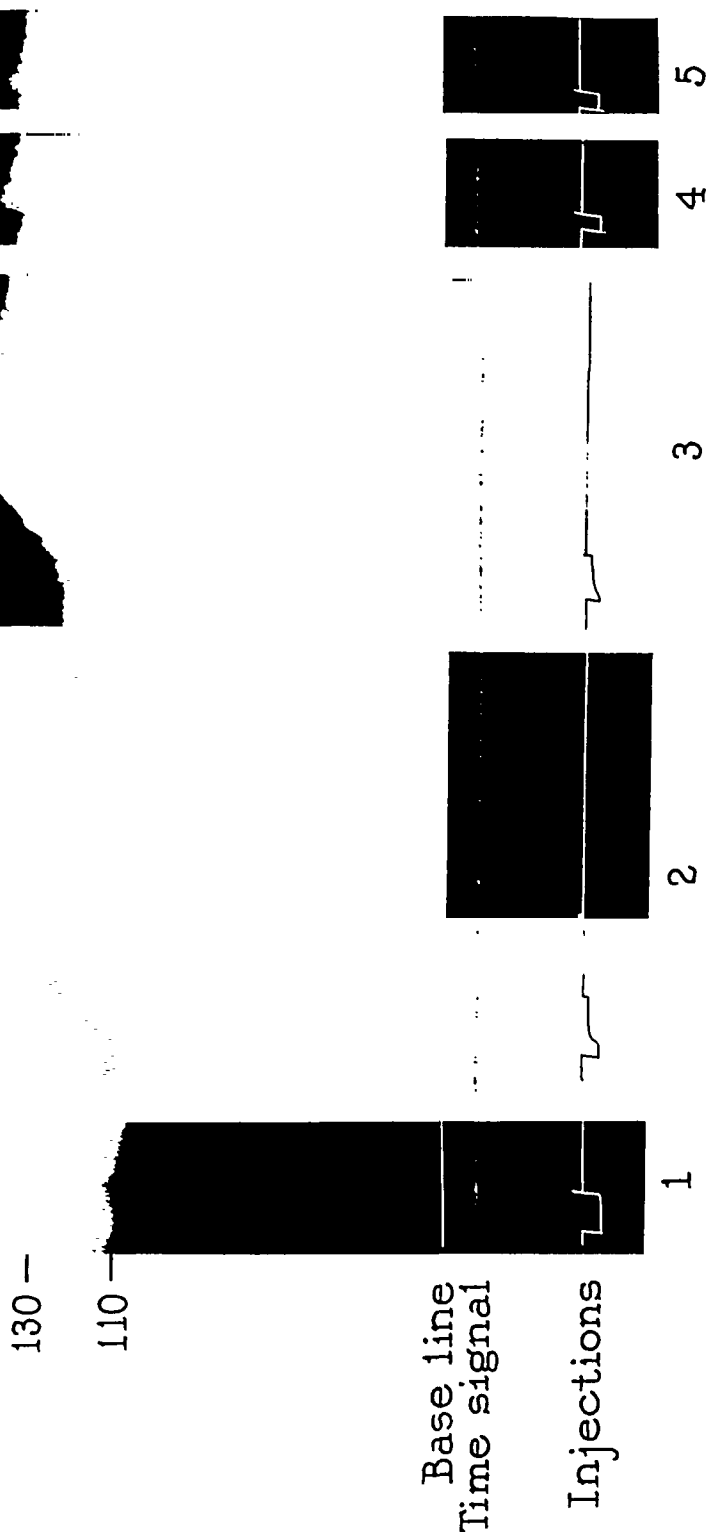


FIG. 2. Comparison of the pressor effects of plasma extract during the reactive and inactive period of testing. Cat under ether anesthesia. (1) Control injection of 5 cc. saline. (2) Extract of 14 cc. of plasma from a moderately severe essential hypertensive patient (B.P. = 202/116). (3) Extract of 14 cc. plasma from severe hypertensive patient (B.P. = 290/130). (4) The ether was cut off ½ hour before this injection, but the animal showed no signs of recovery. The same extract as (1). (5) Same extract as (2). Initial pressure = 116 mm. Hg. Time signal = 10 seconds.

fixed hypnotics are employed as anesthetics. Some hypnotics have the serious disadvantage of damping the vascular responses to pressor extracts to a degree that makes the interpretation of the records difficult. Furthermore, the non-reactive phases (Phase 3) are more difficult to detect.

If extracts are not tested during periods of approximately the same reactivity it is apparent that false results will be obtained. Our problem, namely whether the pressor substance is increased in amount in the plasma of patients suffering from hypertension, could not be answered until this fact was understood. It is not sufficient to test the animal at the beginning of the experiment with extracts of the plasma of normal individuals, and if no response is elicited, assume that any other extracts which produced a rise in pressure are "active." The extracts to be compared must be tested within as short a time interval as is feasible, and in alternating order.

Methods for Estimating the Reactivity of Test Animals to Pressor Extracts

Vascular Response to Peripherally Acting Substances.—The hope that the character of the response of animals to a pressor extract might be predicted by preliminary study of the responses to such powerful pressor and depressor drugs as adrenalin, pitressin, choline, adenylic acid, and histamine, proved illusory. Systematic studies, on a large number of animals, showed conclusively that no parallel or reciprocal relationship existed. These drugs which act primarily on the peripheral blood vessels produced excellent responses when pressor extracts no longer influenced the blood pressure level. Indeed, as will be evident later, the recognition of this fact proved an important key to the understanding of the pharmacology of the pressor substance.

Reactivity as Measured by Carbon Dioxide Inhalation.—The reactivity of the "vasomotor center" was tested from time to time during the course of the experiment by the administration of carbon dioxide-air mixtures.

10 per cent carbon dioxide air mixtures were prepared in a gasometer and 800 cc. portions delivered into a rubber balloon. The gas mixture was administered by means of a No. 10 French catheter, inserted through the rubber tube which delivered the ether, into the tracheal cannula. The tip of the catheter reached

to the bifurcation of the trachea in order to insure delivery of the carbon dioxide to the lungs. The tracheal cannula remained partially open in order not to interfere with spontaneous breathing. The test dose of carbon dioxide-air mixture required 100 seconds for delivery. An alternative method consisted in the insertion of a flutter valve between the gas balloon and the catheter and a second one on the tracheal outlet.³ It was necessary to stop the administration of the anesthetic when this latter system was employed.

During the administration of the test dose of gas the blood pressure began to rise and remained at an elevated level for 2 to 3 minutes, then fell to the original

TABLE I

Comparison in Etherized Cat of the Vascular Response to Carbon Dioxide-Air Mixture and Pressor Extracts

Test substance	Amount	Time	Blood pressure	Magnitude of change
	cc.	p.m.		mm. Hg
NaCl.....	5	1.10	140	0
Ascitic extract.....	5	1.30	122	+ 6
CO ₂ -air.....	800	1.40	116	+ 8
CO ₂ -air.....	800	2.00	114	+12
Ascitic extract.....	5	2.15	100	+16
CO ₂ -air.....	800	2.40	98	+12
Ascitic extract.....	5	3.00	88	+14
CO ₂ -air.....	800	3.30	74	+14
Ascitic extract.....	5	3.45	82	+36
CO ₂ -air.....	800	4.00	112	+32
CO ₂ -air.....	800	4.20	114	+16
Ascitic extract.....	5	4.40	116	+20
CO ₂ -air.....	800	5.10	126	+ 4
Ascitic extract.....	5	5.30	146	+ 6
CO ₂ -air.....	800	5.40	148	0
Pure CO ₂	600	6.20	154	-37

level. In most of our experiments the reactivity of the vascular system was first tested with CO₂-air, then the test dose of plasma extract given.

The results show that when the animal exhibits the greatest response to CO₂-air mixture the response to pressor extract is also marked. Exact parallelism is not always seen. After some time has elapsed (1 to 3 hours) the action of both may become weaker. Finally,

³ The valves can be made conveniently from the rubber of surgical gloves, the edges of the valve being glued together by grippit (a form of rubber cement).

they elicit no further response. Even high concentrations of carbon dioxide are ineffective (Table I). This is the period designated as 4 (Fig. 1).

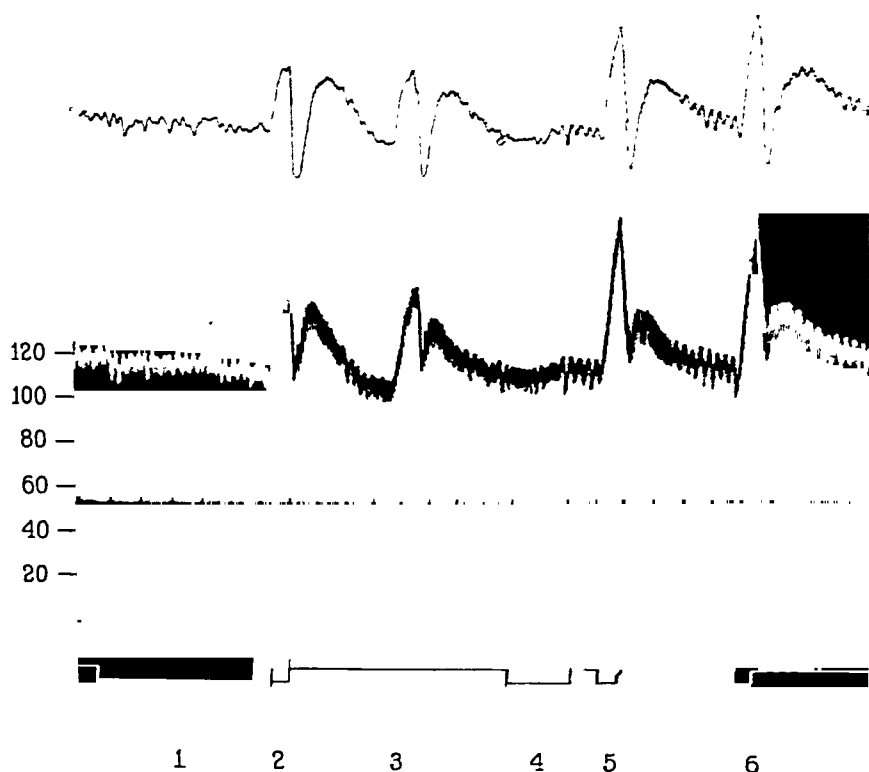


FIG. 3. Increase in response to carotid sinus stimulation following extract injection, without marked change in blood pressure level. Dog anesthetized with 0.10 gm. chloralose per kilo body weight. Upper curve records pressure from peripheral end of the femoral artery. Lower curve is record of the pressure in the central end of the femoral artery. (1) 15 cc. ascitic extract. (2) Sinus stimulation. (3) Sinus stimulation. (4) 15 cc. ascitic extract. (5) and (6) Sinus stimulation. Initial blood pressure = 118 mm. Hg.

Reactivity as Measured by the Response to Carotid Sinus Stimulation.—Occlusion of both common carotid arteries after bilateral vagotomy normally produces a marked rise in systemic blood pressure, due to

stimulation of the carotid sinuses. The nervous connection with the vasomotor center is through the glossopharyngeal nerve. This reflex has been utilized as a means of determining the reactivity of the animal to pressor extract. It has been found that there is a general parallelism between the rise in blood pressure produced by occlusion of the carotid arteries and the responsiveness of the animal to extracts. The parallelism is by no means exact, but is a useful means of determining non-reactive periods.

It has also been possible to demonstrate that the extract in some manner sensitizes the mechanism responsible for the sinus reflex⁴ (Fig. 3).

The rise in blood pressure following sinus stimulation is considerably greater after the injection of extract than before. Extracts which, through some fault in preparation, were depressant to the systemic blood pressure level, invariably markedly reduced the reflex sensitivity. The substance which causes increased sensitivity is contained in the pressor extract alone because ultrafiltrates of plasma had no effect in altering the response to reflex stimulation.

It has not been ascertained whether the rise in blood pressure incident to injection of extract is responsible for the increased sensitivity to the reflex stimulation. Increase in sensitivity has often been observed in experiments where the rise of blood pressure level due to the injected extract was very small.

Other Methods of Control and Precautions Necessary to Observe in Assaying Pressor Extract.—

(a) Rise in blood pressure incident to the intravenous injection of indifferent fluids must always be estimated by the injection of 5 cc. of warm saline. Certain animals are sensitive to changes in blood volume and this, although rare, cautions against the acceptance of one or even two positive results as indicating the presence of pressor substance.

(b) Since in the preparation of plasma extracts the water-soluble elements of the blood become more concentrated than normal, for control purposes artificial extracts were prepared containing three times the concentration of sodium chloride and urea found in normal blood. No clear effect on the blood pressure level was observed from the injection of 5 cc. of this solution.

⁴ We were fortunate in having the direction of Professor Dautrebande of Liège, Belgium, during this part of the work. Dogs under chloralose anesthesia were employed in some of the experiments. These animals, according to Professor Dautrebande's experience, are most suitable for carotid sinus experimentation.

(c) Injections should not be made when the blood pressure is falling at a rapid rate. The pressure fall may be checked by the extract injection and the blood pressure assume a fixed level; it may continue to fall or it may suddenly climb rapidly. None of these responses give any clue as to the potency of the pressor substance in the extract.

(d) Injections must not be attempted when changes either in the rhythm or the amplitude of the pulse are noted on the kymographic record.

(e) The injection must be made slowly and with great evenness, otherwise sharp transient elevations in the pressure curve often occur. In spite of this precaution in some animals a temporary rise in pressure cannot be avoided even when small amounts of saline are injected.

(f) In general it has been found undesirable to test extracts in animals in which the blood pressure is exceptionally high or low. The usual level at which our tests were made was about 100 to 140 mm. of mercury.

(g) The respiration must be moderately slow and regular and in no way hindered by the respirator. Usually, during periods when the natural breathing had stopped, the vascular responses were at first very sluggish and if anoxemia supervened they became highly irregular.

(h) In order to compare the potency of two extracts, the injections should be given within as short a space of time as is feasible, to guard against changes in the sensitivity of the animal to the extract. It has been found best to administer the smallest doses of the extract that will produce a rise in blood pressure which is definite, and to follow this injection with the extract with which it is to be compared as soon as the pressure has returned to the original level. The first extract is then again injected to make sure that the animal is still reactive. While this method demands much time and relatively large amounts of extract it has seemed the only one which is reliable.

Pharmacology of Pressor Extracts

The typical pressor response consists in a relatively slow rise in the level of the blood pressure and the steady maintenance of the elevated level for some time (Fig. 4). No preliminary depression of the level of blood pressure occurs.

The Action of Plasma and Ascitic Fluid Extracts on Cats Treated with Ergotoxine.—In order to determine whether the response to pressor extracts could be influenced by ergotoxine, cats under ethyl urethane anesthesia were first tested with 1 cc. of 1:100,000 adrenalin followed by 5 cc. of pressor extract as control. Now, 1 mg. of ergotoxine tartrate (gynergen, Sandoz) was injected intravenously and after 2 minutes the animal was retested with adrenalin and extract. It could be shown that although reversal of the adrenalin pressor effect had

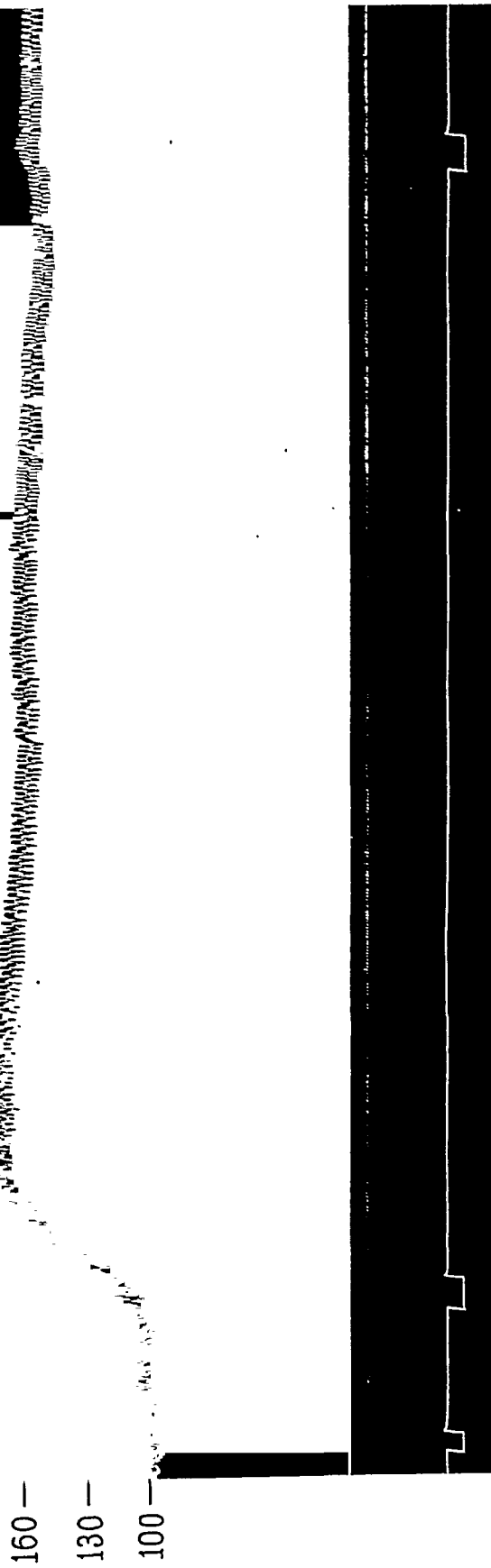


FIG. 4. Example of a very active response to plasma extract from a patient with toxemia of pregnancy and hypertension. (1) Saline 5 cc. (2) Extract of 10 cc. plasma from patient (B.P. = 140/104). (3) Same extract boiled 3 minutes. Upper tracing represents renal volume. Experiment 124 (3-5). Initial pressure = 100 mm. Hg. Time markings = 10 seconds.

been produced the rise in blood pressure resulting from plasma or ascitic fluid extract was not altogether abolished. After large doses of ergotoxine the central nervous system of the animal appeared depressed as shown by the fact that the responses to occlusion of the carotid arteries were very weak. When this occurred the pressor extracts were also almost ineffective.

Influence of Cocaine and Atropine on Response to Extracts.—It is well known that cocaine "sensitizes" the vascular response to adrenalin. Therefore it seemed desirable to ascertain whether this drug would influence the response to pressor extracts.

Cocaine (2 mg. per kilo) was injected intravenously into the cat under ethyl urethane anesthesia after the vascular response to adrenalin solution (1.5 cc. of 1:100,000) and to 5 cc. of pressor extract had been determined. The same amount of adrenalin was again injected to be certain that the magnitude of the response had increased and the pressor extract then injected. Adrenalin raised the pressure 10 mm. Hg before cocaine was given and 30 mm. Hg afterward. The extract raised the pressure level 18 mm. Hg and after cocaine 21 mm. It seems safe, therefore, to conclude that cocaine in the dosage we have employed does not affect the response to extract to any marked degree. No evident alteration in the responsiveness of the vascular system to carotid sinus stimulation was observed as the result of cocaine injection.

To ascertain the effect of atropine on the pressor response both cats and rabbits were given atropine by vein until the characteristic reversal of the action of choline from depressor to pressor had occurred. Active plasma extracts were found quite as active after atropinization as before.

Effect of Curare on the Pressor Response.—Animals under ether anesthesia were curarized by repeated intravenous injections of 3 mg. doses of curare until normal respiration was completely paralyzed. Artificial respiration was now begun.

The response to pressor extract was sluggish in such animals. As there seemed no advantages over the ordinary method of assay and there were definite disadvantages, the procedure was not further employed.

Response of the Kidneys to Pressor Extracts.—In order to ascertain what effect the pressor extracts exerted on the kidneys' volume, the

right kidney of the test animal was placed within a Livingston glass oncometer. The volume changes were registered by a small Brodie bellows.

Observation of many curves from such experiments convinces one of the extraordinary autonomy of the kidneys' circulation. Under apparently identical circumstances the kidneys may shrink or swell following the injection of certain substances. In general, the active plasma extracts tended to cause the renal volume to increase slightly though the opposite effect may be observed when the blood pressure rise is great. Without measurements of the blood flow through the kidney it is not possible to state whether vasodilatation or constriction had occurred. As has been pointed out by Richards and Plant (52) epinephrin may cause the renal volume to increase but simultaneously constrict the efferent arterioles, thus causing increased intraglomerular pressure.

As the result of pithing animals the renal volume curve follows accurately that of the carotid blood pressure, regardless of the agent employed to produce blood pressure changes (Fig. 5).

Changes in Peripheral Blood Supply as Measured Oncometrically.—Changes in peripheral blood supply of the hind leg were measured by an oncometer connected with a Brodie bellows. The results of measurements on sixteen animals show that ordinarily a moderate constriction in the leg volume occurs following the injection of plasma extracts. The constriction is initiated rather more slowly than the change in renal volume but it also lasts somewhat longer.

Response to Pressor Extract after Evisceration.—It could be shown by the oncometric method that, while both the renal and the peripheral blood vessels constricted as the result of the injection of the pressor extract, the action was not powerful. It seemed possible, therefore, that the splanchnic vessels constituted a not inconsiderable portion of the constricted area during the rise in systemic blood pressure.

After preliminary determination of the response to 5 cc. of plasma extract and the reactivity of the carotid sinus reflex, the anesthetized animal was eviscerated in the usual manner. The responses were again tested shortly after the blood pressure had steadied itself following the operation. An example may be given to illustrate the results of this procedure. Before evisceration the pressor response to the extract consisted in a rise in blood pressure of 22 mm. Hg, and



1 2 3 4 5 6 7 8 9 10 11

FIG. 5. Action of plasma from a nephritic patient on sensitivity of the pithed cat to epinephrin. The upper line represents renal volume; the second line, arterial blood pressure; and the third is the pneumographic record of respiration. No anesthetic. (1) Epinephrin 2 cc. (2) Pitressin 1.5 cc. of 1:25 dilution. (3) Epinephrin 2 cc. (4) 2 cc. plasma of severe nephritic patient with hypertension. (5) Epinephrin. (6) Pitressin. (7) Extract of 10 cc. plasma of same patient. (8) Epinephrin. (9) Nephritic plasma 5 cc. (No. 2). (10) NaCl 5 cc. (11) Pitressin. Experiment 116 (27-34). Blood pressure = 40 mm. Hg.

that due to carotid sinus stimulation was 16 mm. Hg. After evisceration the extract raised the blood pressure level 10 mm. Hg and the carotid sinus stimulation, 24 mm. Hg. The systemic blood pressure fell from 108 mm. Hg to 84 mm. Hg as the result of the operation.

The findings suggest that constriction of the vessels of the splanchnic area plays a large part in the elevation of blood pressure level resulting from the injection of pressor extract.

Effect of Adrenalectomy on the Effect of Pressor Extracts.—The possibility that the pressor action of plasma extracts might be due to stimulation of the adrenal gland causing the secretion of adrenalin was studied by bilateral removal of these glands. Recent experiments have shown that certain pharmacological agents do in fact owe at least part of their action to the increased liberation of epinephrin into the circulation.

After preliminary testing of the potency of an extract on a cat under ether anesthesia, the adrenal glands were either tied off or extirpated. The lumbar approach to the glands was employed. The extracts were again tested within 10 minutes and 1 hour after adrenalectomy.

The results from ten such experiments indicate that rather than a decreased response, the opposite was found. The increase, however, was relatively small, and in view of the spontaneous alterations in reactivity of the animal it does not seem justifiable to conclude that adrenalectomy causes sensitization to pressor extracts.

The Effect of Bilateral Vagotomy.—Extracts tested before and after bilateral vagotomy did not exhibit any characteristic difference. When the vagi were intact, spontaneous fluctuations in the level of the blood pressure were more apt to appear, consequently in most of our experiments this operation was performed.

Does the Animal Develop Immunity to the Pressor Action of Plasma Extracts?—Since it is well known that repeated injection of extracts of the posterior lobe of the pituitary result in the gradual development of immunity in animals to further vascular stimulation, it seemed desirable to ascertain if this were true of plasma extracts. It was found that the second response is just as great and often greater than the first. Indeed, the pressure level can be built up step-wise by the repeated injection of extract. As the blood pressure reaches levels above 160 mm. Hg the response to additional extract is likely to be

100—
80—
60—
40—
20—

88

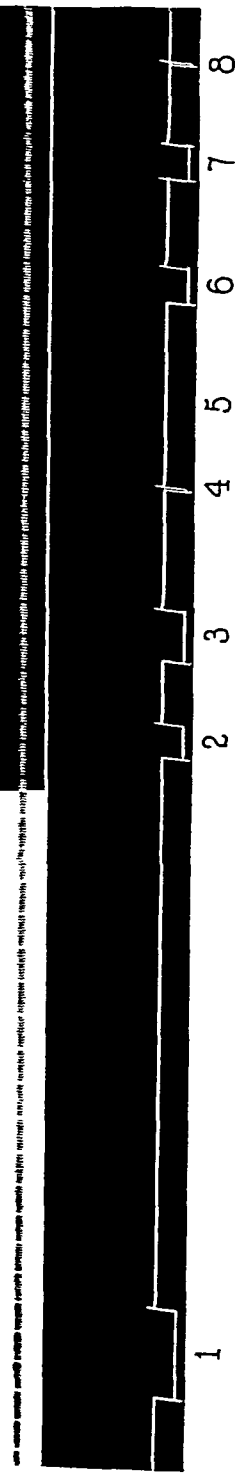


FIG. 6. Pressor response to 5 cc. of extract of 10 cc. ascitic fluid. Before and after pithing in cat under ethyl urethane anesthesia. (1) 5 cc. extract injected. (2) After pithing, 5 cc. extract injected. (3) 5 cc. normal saline. (4) and (5) Adrenalin 0.5 cc. 1:200,000. (6) 5 cc. extract. (7) Same. (8) Adrenalin 1 cc. 1:200,000. Initial pressure before pithing = 100 mm. Hg. After pithing pressure = 44 mm. Hg.

reduced. While "immunity" may develop in the sense that the animal no longer responds to extract this has seemed to be due to failure of the central nervous system because, coincident with the "immunity," the animal fails to respond to inspired CO₂-air mixture and usually to carotid sinus stimulation.

Effect of Progressive Destruction of the Central Nervous System.—The great variability of response noted in the test animal suggested that the substance or substances with which we were dealing might depend for their action on the cooperation of some portion of the central nervous system. It was assumed that during the initial period (Period 1), the lack of response was due to a temporary paralysis of the central nervous system, caused by anesthesia, when perforce an excess of ether was ordinarily employed. The sluggish reactions again, in Period 3, suggest that a more complete paralysis had occurred.

In order therefore to determine whether the central nervous system is an essential part of the mechanism of the pressor response, anesthetized cats were pithed to the second lumbar segment through a trephine hole in the skull, after tying off both carotid arteries. These animals responded markedly and regularly to adrenalin and pitressin. Such preparations are more sensitive to peripherally acting pressor substances than those in which the central nervous system is intact, provided the artificial respiration is properly regulated.

It could be shown that no response whatever was elicited by the injection of active pressor extracts (Fig. 6) into these pithed animals.

In order to localize more precisely the portion of the nervous system involved in the pressor response, the brain was removed at various levels in anesthetized, vagotomized cats, by means of the Sherrington guillotine. The action of pressor extract was not unusual until the cut passed through the brain somewhat above the bony tentorium. At and below this level, the responsiveness of the animal was slightly increased. However, section just below the tentorium abolished all effect of extracts.

Action on Unanesthetized Animals.—That the pharmacological action of many substances is markedly influenced by the anesthetic employed during the testing, is well known. It seemed important, therefore, to eliminate this factor as an influence in the action of the pressor extract.

For this purpose rabbits were found most satisfactory since they would lie quietly while the blood pressure was being taken. The method of Koch and Mies (53) was used to measure the blood pressure. This consists essentially in the preliminary operative transplantation of the carotid artery above the muscles of the neck of the anesthetized animal in such a way that a metal chamber containing a glass window can be clamped over the artery. One side of the chamber is covered by rubber membrane, and it is on this membrane that the artery is placed; that is, between the membrane and the glass window. Air pressure is applied against the membrane until the column of blood in the artery is just obliterated, and the pressure is then read from a mercury manometer. The transplantation of the artery was performed a day or two before the experiment. Often 0.5 cc. of 50 per cent ethyl urethane was given subcutaneously 15 minutes before the experiment in order to insure that the animal would remain quiet. By gentle handling we have had no difficulties. After applying the chamber it is well to wait 15 minutes before taking readings. A control period of 30 minutes was carried out, taking readings every 5 minutes to observe the range of spontaneous fluctuation in blood pressure. Our experience has been that most animals maintain under the conditions of the experiment a blood pressure of about 115 to 135 mm. of Hg without wide fluctuation.

Under the influence of ether, amytal, or ethyl urethane, rabbits have been found but moderately responsive to pressor extracts which have been found highly active in cats. The intravenous injection in non-anesthetized animals (ear vein) of 5 cc. portions of extract induced blood pressure elevation of 15 to 22 mm. of Hg. which appeared almost immediately after injection, and lasted 3 to 8 minutes. The same amount of normal saline produced practically no effect on the blood pressure.

This procedure has been controlled by the more usual method in which the carotid artery is cannulated and the pressure recorded graphically. The vessel was prepared, the cannula inserted under light ether anesthesia, and as soon as the operation was complete the cannula was stoppered. The animal was then allowed to recover from the anesthesia. 1 hour later blood pressure level was recorded. Records taken over 15 minute periods exhibited blood pressure of remarkable constancy. Pressor extracts ordinarily gave responses some 5 to 10 mm. of Hg greater than the same extracts tested on the same animal while anesthetized with ether.

Miscellaneous Observations Related to the Problem of Pressor Substances in Blood

Ultrafiltrates of Plasma Tested on Non-Anesthetized Animals.—In view of the observation of Anselmino and Hoffmann (15-17) that

ultrafiltrates of plasma or serum of blood from patients suffering from eclampsia, in whom the blood pressure was greater than 180 mm. of Hg, produced a rise in blood pressure of rabbits, it seemed desirable to test ultrafiltrates derived from the plasma of cases of essential and nephritic hypertension by the same method. The Koch-Mies method (53) has been used in the manner suggested by Anselmino and Hoffmann.⁵

After the control period, during which we convinced ourselves that the blood pressure of the animal was constant, 10 cc. portions of neutralized (to phenolphthalein) ultrafiltrate were injected subcutaneously into each flank of the animal and the injection site well massaged. Pressure readings were then taken every 5 minutes for a period of 30 minutes. Four plasma extracts from cases of essential and malignant hypertension (blood pressures ranging from 220 to 290 mm. systolic and 120 to 160 diastolic), and four cases of hemorrhagic Bright's disease with blood pressures from 195 to 250 systolic and 128 to 145 diastolic) were examined.

In no experiment was any effect on the blood pressure of the rabbit observed. The conclusion seems justified that while the conditions were excellent for a positive outcome of the experiment, the results were uniformly negative.

Ultrafiltrates of Plasma Tested on Anesthetized Animals.—Ultrafiltrates of plasma exhibited no marked action on the blood pressure level of anesthetized cats when injected intravenously in amounts up to 20 cc. While it is possible that a small quantity of pressor substance may pass the filter membrane, the fact that almost 90 per cent of the active material may be extracted by alcohol from the non-filterable residue, clearly indicates that the amount which is dialyzable is very small. Alteration of the pH of the plasma before ultrafiltration did not change this result.

The extract of plasma prepared by the alcohol method as contrasted with native plasma was easily passed through the filter membrane without loss of activity.

Extracts of Blood Corpuscles.—Extracts of red blood cells prepared in the same manner as plasma extracts generally depress the blood pressure level. This is not always true as rare specimens have been found which produced slight pressor action and others have had none.

⁵ We were fortunate in having the aid of Dr. Anselmino.

120 samples of red blood cells from patients suffering from essential hypertension, hemorrhagic Bright's disease, with and without hypertension, and red cells from normal individuals, have been compared. Analysis of the many graphs so obtained reveals no effect which is characteristic for any especial group of patients. It does not, therefore, seem necessary to present the detailed data.

Extracts of blood cells which have been hemolyzed by the addition of distilled water and then added to alcohol (20 cc. red cells + 20 cc. water in 380 cc. alcohol 95 per cent) and the extract prepared in the manner described for plasma also exhibited no characteristic difference from red cells of the same blood specimen in which hemolysis had been scrupulously avoided.

The amount of depressor substance in very fresh red blood cells (in alcohol within 5 minutes of venipuncture) is ordinarily not great. Extracts which correspond to 2 cc. of centrifuged cells may depress the blood pressure level of a 3.5 kilo cat 12 to 20 mm. of mercury. Allowing corpuscles to stand before addition to alcohol longer than 2 hours may cause to be liberated most powerful depressor substances. The injection of such an extract, corresponding to 2 cc. of corpuscles, may be quickly fatal to the animal. Atropine does not alter the depressor action of these extracts.

CLINICAL RESULTS

Since it could be shown that a pressor substance was extractable from the blood of man, it was of importance to ascertain whether the amount of this substance was altered in patients suffering from hypertension regardless of the pathogenesis of the symptom.

165 experiments have been performed, and from these data it seems almost certain that there is no significant correlation between the blood pressure response in the cat and the blood pressure of the patient from whom the plasma was obtained. Suffice it to say that the blood of 33 normal individuals, 8 cases of latent hemorrhagic Bright's disease, 12 cases of acute hemorrhagic Bright's disease, 11 cases of chronic active hemorrhagic Bright's disease, 19 cases of terminal nephritis, 8 cases of nephrosis or the nephrotic stage of Bright's disease, 38 cases of essential hypertension, 11 cases of toxemia of pregnancy, 1 case of cirrhosis of the liver without hypertension, and 2 cases of severe eclampsia have

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CLINICAL RESULTS

Since it could be shown that a pressor substance was extractable from the blood of man, it was of importance to ascertain whether the amount of this substance was altered in patients suffering from hypertension regardless of the pathogenesis of the symptom.

165 experiments have been performed, and from these data it seems almost certain that there is no significant correlation between the blood pressure response in the cat and the blood pressure of the patient from whom the plasma was obtained. Suffice it to say that the blood of 33 normal individuals, 8 cases of latent hemorrhagic Bright's disease, 12 cases of acute hemorrhagic Bright's disease, 11 cases of chronic active hemorrhagic Bright's disease, 19 cases of terminal nephritis, 8 cases of nephrosis or the nephrotic stage of Bright's disease, 38 cases of essential hypertension, 11 cases of toxemia of pregnancy, 1 case of cirrhosis of the liver without hypertension, and 2 cases of severe eclampsia have

been examined.⁶ From many of these patients blood samples have been taken at intervals of a week for periods upwards of 16 months. During this time marked variations in the level of the patient's blood pressure had occurred (Fig. 7). Because of inherent difficulties in the method of assay, it was necessary to secure data which would be

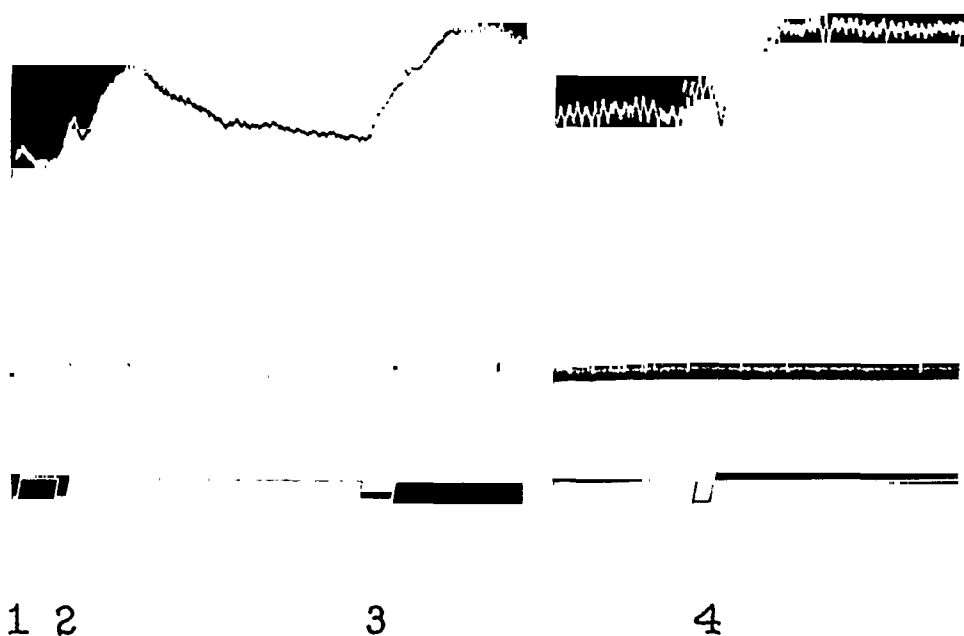


FIG. 7. Comparison of extract of nephritic plasma with ascitic fluid extract. (1) Saline 5 cc. injected. (2) Extract of 14 cc. plasma from severe nephritic patient with hypertension (B.P. = 200/122). (3) Extract of 14 cc. of ascitic fluid from patient with cirrhosis of liver without hypertension. Experiment 166 (8-9). (4) Extract of 10 cc. plasma from the patient with liver cirrhosis. Experiment 163 (No. 15). Initial pressure = 84 mm. Hg.

statistically significant. This goal, we believe, has been achieved, and it must be fairly stated that no correlation appears to exist between the amount of pressor substance and the height of the blood pressure.

⁶ The blood samples from the cases of toxemia of pregnancy and eclampsia were furnished by Dr. E. Stander and Dr. L. Maclean of the Cornell Woman's clinic. We are grateful to Drs. Stander and Maclean for their courtesy.

It has been possible to examine blood from cases⁷ of the recently described pituitary basophilism. Three of these cases had rather marked hypertension⁸ and in the other two the pressure averaged 140 to 150 mm. Hg systolic and 90 to 94 diastolic. All the patients exhibited more or less typically the signs and symptoms of this disease as set forth by Cushing. No characteristic difference was observed in the ultrafiltrates or extracts of plasma from these patients as compared with extracts of normal blood.

As an incidental observation it seems worth recording that from a limited number (3) of patients suffering from convulsive uremia extracts of blood plasma seemed peculiarly toxic when injected into a cat. Instead of the usual rise in blood pressure, a precipitous fall occurred, causing the death of the animal. It should be emphasized that these patients were in the terminal stage of hemorrhagic Bright's disease and not examples of the so called pseudo-uremia.

Cerebrospinal fluids from nineteen assorted cases of nephritic and essential hypertension have also been examined. Pressor substance is present in at least as large amounts as in plasma. Injection of fresh spinal fluid from a variety of patients suffering from hypertension failed to show the presence of any appreciable amount of free pressor substance when tested either on anesthetized animals with the central nervous system intact, or on pithed cats. The possibility that unbound secretion of the posterior pituitary body is contained in the spinal fluid in amounts which might be considered significant in the pathogenesis of hypertension, has not been demonstrated. This statement must be qualified to a certain extent in that the ventricular fluid from one case of adrenal tumor contained a powerful pressor substance with properties somewhat different from those of the pressor extracts of plasma. Spinal fluid of one patient with a severe malignant hypertension also was powerfully vaso-active.

DISCUSSION

The pressor substance evidently is widely distributed since it has been found in human plasma, ascitic and cerebrospinal fluids. Corpuscles seem to contain little or none of it because alcohol extracts

⁷ Through the kindness of Dr. Harvey Cushing and Dr. B. S. Oppenheimer.

⁸ Dr. Henry Turner, Oklahoma City, very kindly sent samples of blood from one such case.

either do not influence or strongly depress the level of the blood pressure of cats. That it is not free in these fluids follows from the fact that ultrafiltration yields a protein-free filtrate which has no vaso-activity. It seems probable that the substance is bound to the plasma colloids but the union must be loose, to be split by such mild treatment as that with cold alcohol. The active substance is water-soluble and behaves not unlike an organic base.

The pharmacological properties of the pressor substance appear in some respects unique. The evidence, in the first place, gathered from comparison with drugs acting on peripheral arteries such as adrenalin, choline, histamine, and adenosine, does not lend support to the view that its action resembles that of these other substances. The effect of ergotoxine and cocaine on the response to pressor extract also demonstrates the dissimilarity in action.

The second group of data indicates that the functional intactness of the central nervous system is essential in order that pressor responses be obtained from the extract under investigation. The most cogent of these proofs consists in the demonstration that if the brain is removed close to the bony tentorium, the pressor response is undisturbed, conversely the response is completely abolished on pithing or cutting the cord below the medulla. Anesthesia, furthermore, prolonged to the point where inhalation of carbon dioxide-air mixtures or stimulation of the carotid sinus no longer elevate the blood pressure, also inhibits its action. The unanesthetized animal (rabbit) is, in fact, more responsive than the anesthetized. It is not improbable that some substance in the extract sensitizes the mechanism responsible for the carotid sinus reflex, for after injection of extract the reflex is considerably more active than before.

A third group of more miscellaneous data indicates that the splanchnic area is actively involved in the vascular constriction. The evidence consists largely in the demonstration that evisceration strongly depresses the response and oncometric measurement of the leg and renal volume shows relatively little constriction when marked elevation of the level of pressure has occurred. Stimulation of the adrenals to secrete more epinephrin apparently plays little or no part in the mechanism because bilateral adrenalectomy does not influence the result. An assay of the potency of the extract has been difficult

because the degree of response depends on the functional state of the nervous system. To some degree success has been attained in predicting the response of the animal to pressor extract by relying on the reflex from the carotid sinus and on that from stimulation by carbon dioxide. No evidence has been found indicating that there is an increase in the amount of pressor substance in hypertensive states of varied pathogenesis.

This investigation was undertaken to ascertain whether, by direct methods which involve as slight chemical manipulations as possible, a single substance or a group of substances is extractable from blood which causes or is associated with arterial hypertension in man. There are numerous possibilities. Such substances, present in normal blood, may be increased in the blood of hypertensive patients; they may be present only in hypertensive states; though constant in amount, the sensitivity of the vascular system may be so increased that its response is pathological; the amount bound to plasma colloid may remain constant though the amount liberated, due to the activity of the reacting cells (*Erfolgsorgan*), may be greater than normal; and, finally, they may be set free into the blood stream and either be quickly destroyed or inactivated. While it may be necessary to reckon with these manifold possibilities this investigation has been confined to the effort to demonstrate the presence of pressor substances in circulating blood and their quantitative aspects in relation to the hypertensive state in man.

CONCLUSIONS

1. Extracts of human blood plasma, ascitic and cerebrospinal fluids have been shown to contain a substance or substances which have a prolonged and powerful pressor action when injected into test animals.

2. The chemical properties of the substance suggest those of an organic base. It is extracted with alcohol, soluble in water and acetone, extracted from water by chloroform, and probably is but slightly heat-stable. The plasma colloids seem to hold the substance in a bound state since it does not appear in the ultrafiltrate and is liberated on coagulation of the colloids by alcohol. Coagulation alone of the blood does not cause the substance to be formed.

3. Its action suggests that its pressor effect is brought about by

mediation of the central nervous system. This inference was drawn from the following observations. (a) The functional intactness of the central nervous system is essential in order that pressor responses be obtained. Unanesthetized animals exhibit greater vascular responses than do anesthetized. (b) Pithing animals completely abolishes the response. Progressive ablation of the brain to the level of the hind brain does not alter the response, but below this level, injury abolishes the activity of the extract. (c) Some substance in the extract sensitizes the mechanism responsible for the carotid sinus reflex. (d) There is no parallelism between the response to peripherally acting drugs and pressor extracts. (e) Removal of the adrenal glands does not affect its character.

4. The rise in blood pressure appears to be due especially to constriction of the arteries in the splanchnic region.

5. Assay of the pressor extracts is made difficult because of the dependence of the vascular response on the functional state of the central nervous system. The carotid sinus reflex and stimulation with carbon dioxide-air mixtures have proved most useful means for the estimation of this functional state. It has been pointed out that the vascular responses to extract, stimulation of the carotid sinus, and inhalation of carbon dioxide-air vary greatly during the course of an experiment on anesthetized animals. This natural history of the vascular responses has been described.

6. No evidence has been produced by the method employed that the amount of this pressor substance is increased in the blood or spinal fluid of patients with hypertension of varied pathogenesis (nephritic hypertension, essential hypertension, malignant hypertension, eclampsia, and pituitary basophilism).

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OBSERVATIONS ON THE DEPRESSOR EXTRACTS OF HUMAN BLOOD AND ON THE VASCULAR ACTION OF EXTRACTS OF RABBIT AND DOG BLOOD*

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(Received for publication, October 12, 1934)

It has been shown that alcoholic extracts of human body fluids contain a substance or a group of substances which, when injected into cats or rabbits, markedly elevate the blood pressure level.¹ The search for pressor extracts has been extended to other species with results which are at variance with those exhibited by human blood plasma. It was shown that extracts of plasma of rabbits always produced depression of the level of blood pressure of anesthetized cats, whereas extracts of dog blood had but the slightest degree of this action.

While extracts of fresh human plasma exhibit only pressor action it was found that when whole blood, plasma, ascitic or spinal fluid was allowed to stand for more than an hour or two, or if hemolysis occurred, the action of the extract from any one of these fluids was invariably powerfully depressor. We have attempted to learn more concerning this action by comparing the simultaneous effects of these extracts on the volume of the leg, the renal volume, and the blood pressure of the animal, with those of other extracts of tissue or adequately characterized depressor substances.

Method

The preparation of extracts of whole blood, plasma, and serum has been described in the previous paper. The method consists essentially in the precipitation of heparinized blood or plasma with alcohol, filtration, and removal of the alcohol at low temperature with the aid of a vacuum. The excess lipid

* Reported at a meeting of the American Society for Clinical Investigation April 30, 1934.

¹ Page, I. H., *J. Exp. Med.*, 1935, 61, 67.

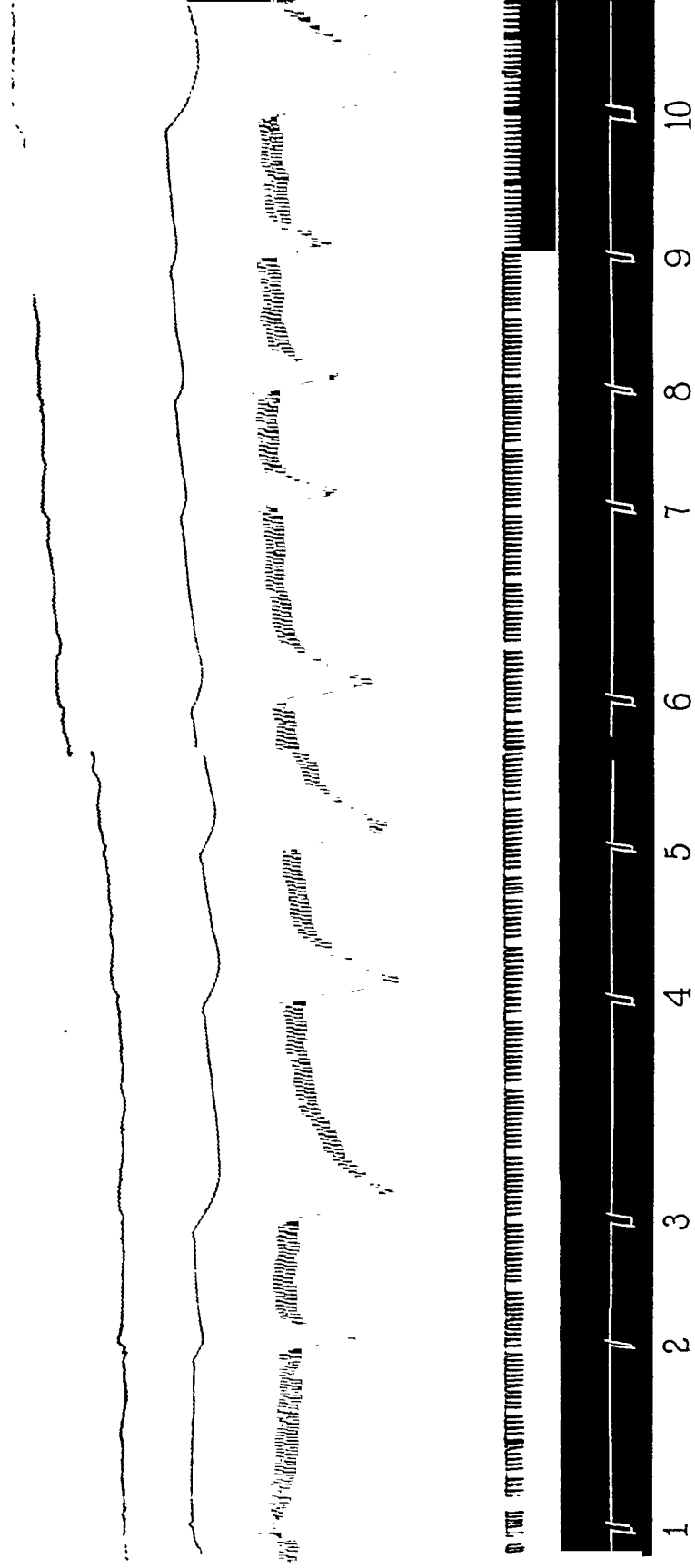


FIG. 1. Comparison of plasma, corpuscle, and liver extract of rabbit with choline and histamine. Upper tracing = leg volume; middle = kidney volume; lower = carotid blood pressure. Time intervals = 10 seconds. (1) Saline 5 cc. (2) Choline 1.5 mg. (3) Extract of 3 cc plasma. (4) Extract of 3 cc. corpuscles. (5) Extract of 3 cc. plasma. (6) Extract of 3 cc. corpuscles. (7) Choline 1.5 mg. (8) Extract of liver. (9) Same. (10) Histamine 0.001 mg.

was then removed by chilling the extract. Ultrafiltrates were prepared by filtration through collodion membranes at high pressure. Extracts of tissue were made by boiling the fresh minced tissue in 0.1 per cent acetic acid for 3 minutes, filtering, and adjusting the filtrate to pH 7.4 just before injection into the animal.

Cats anesthetized with either ether or ethyl urethane were employed for the testing. Vagotomy was performed, the right kidney inserted into a Livingston oncometer, the left leg into a plethysmograph, and either the right carotid or the right femoral artery cannulated for recording the blood pressure.

RESULTS

Whole blood, plasma, serum, or corpuscles of rabbits, whether the blood was removed by puncture of the heart or by puncture of a peripheral artery or vein, yielded extracts which were powerfully depressor in action (Fig. 1). The renal volume is markedly reduced and the leg vessels dilated simultaneously with the fall in pressure. This action is unaltered by preliminary atropinization of the animal. The depressor action of plasma extracts differs from that of choline not only in that it is not abolished by atropine but also in that the constriction of the kidneys is quantitatively much more powerful and the peripheral dilatation considerably weaker. Apparently choline contributes little or nothing to the depressor action of plasma extracts.

The powerful constrictor action in the kidneys is one of the most characteristic features of either corpuscle or plasma extracts of rabbit blood. From time to time, reversal of the usual depressor effect to a pressor one is observed. Even in these cases where the pressure rises instead of falling, the typical constriction of the kidneys is seen. This action on the kidneys resembles closely that resulting from the injection of adenylic acid. Histamine also produces constriction of the kidney but with the same degree of reduction in blood pressure, adenylic acid has a more powerful constrictor action.

Ultrafiltrates of rabbit blood plasma are actively depressant and also constrict the kidneys. Human plasma ultrafiltrates are inactive.

Extracts of plasma from dogs' blood plasma, whether arterial or venous, have almost no effect on the blood pressure of an etherized cat. Occasionally slight depressor action is observed. Extracts of whole blood, taken directly from the cannulated vessel into alcohol, exhibit very slight depressor action, while corpuscle extracts are moderately active depressants. The ultrafiltrates from plasma are uniformly without vascular action.

Comparison of Depressor Extracts of Human Blood with Other Depressor Substances and Extracts

Human blood plasma differs from that of the rabbit and dog in yielding extracts which are pressor. However, if the blood is allowed to stand at room temperature for an hour or two, or to hemolyze, the extracts are powerfully depressor. Human ascitic and spinal fluid also yield pressor extracts when fresh, but if allowed to stand the extract yielded is depressor.

Inspection of many graphic records shows that plasma extracts which exhibit depressor action produce marked renal constriction and relatively insignificant changes in the leg volume. In this respect plasma extracts resemble the action of corpuscle extracts.

Human corpuscle extracts, whether the extract has been prepared from intact or previously hemolyzed cells, are usually moderately strongly depressor. The depressor action is accompanied by marked constriction of the kidneys with an insignificant change in the leg volume. It is possible that the red cells liberate depressor substances into the plasma on standing. Many of our experiments indicate that this is true.

Comparison has been made of the action of depressor extracts of plasma with that of acetic acid extracts of kidney, liver, and histamine, adenosine,² adenylic acid, and choline (Fig. 2). Ordinarily histamine depresses the level of the blood pressure to an extent out of proportion to its action on the renal volume when compared with plasma extracts. Adenosine also has a more marked action on the level of blood pressure than on renal volume but adenylic acid may exhibit extreme constrictor action on the kidneys without markedly affecting the level of the blood pressure.

Choline, on the other hand, may depress the blood pressure to the same extent as plasma extract without at the same time producing marked renal volume reduction. Peripheral dilatation is especially active. The effect of extracts of liver and kidney closely resembles that of depressor plasma extracts, especially as regards their powerful constrictor action on the kidneys.

² The adenosine and adenylic acid were kindly supplied by Dr. P. A. Levene and Dr. S. A. Harris.

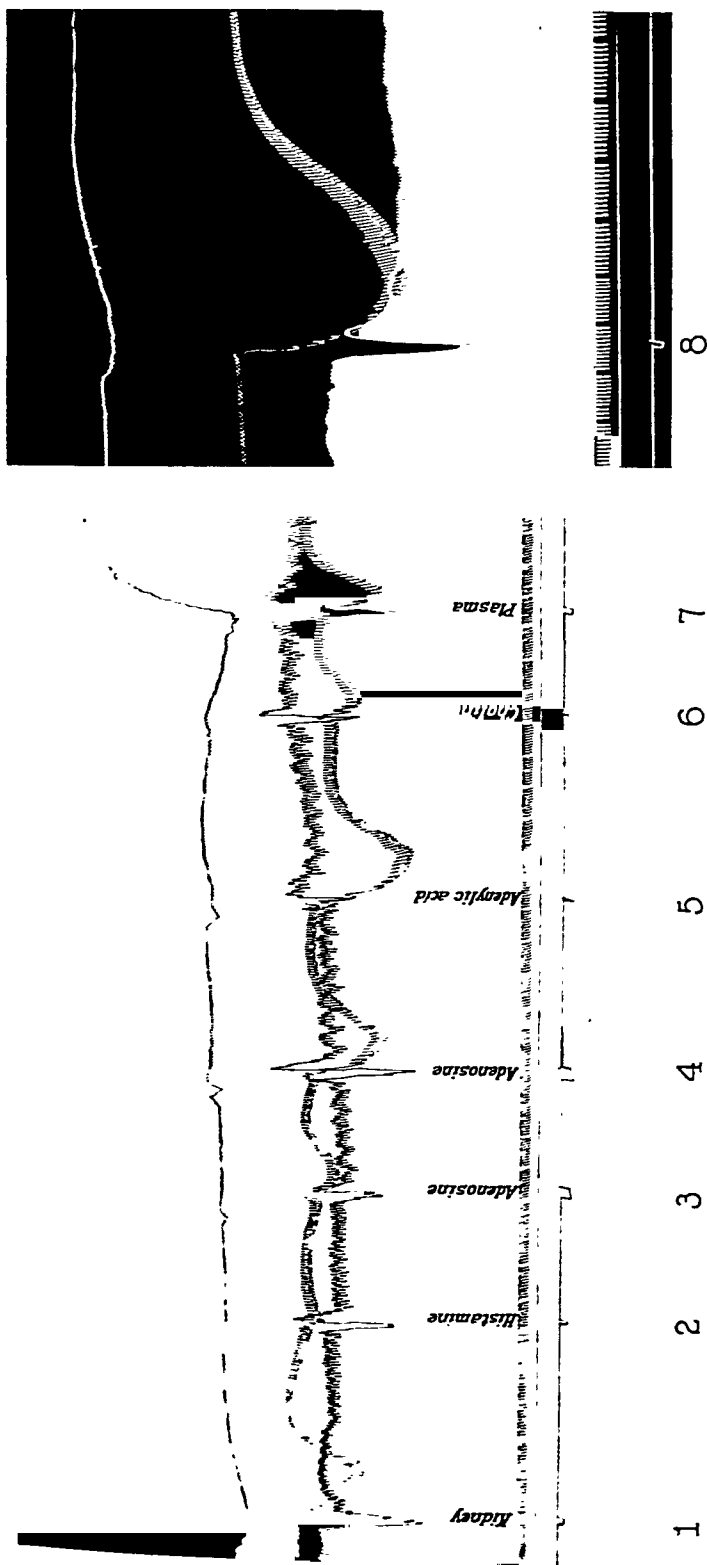


Fig. 2. Comparison of the action of various depressor substances with depressor extracts of plasma. Upper curve = leg volume; middle = renal volume; lower = carotid blood pressure. Etherized cat. Time intervals = 10 seconds. (1) Acetic acid extract of rabbit kidney. (2) Histamine 0.0007 gm. (3) Adenosine 6 mg. (4) Adenosine 10 mg. (5) Adenylic acid 2 mg. (6) Choline 4 mg. (7) Plasma extract equivalent to 4 cc. plasma. (8) Acetic acid extract of rabbit liver.

CONCLUSIONS

1. Alcoholic extracts of the plasma of rabbits obtained from arterial or venous blood differ from those of human plasma in having a marked depressor action on the blood pressure of anesthetized cats. This action is unantagonized on atropinization. Extracts of the plasma of dogs, on the other hand, are almost without vascular effect.

2. Ultrafiltrates of the plasma of rabbits are actively vasodepressor, while those of human and dog plasma are without action.

3. Extracts of the plasma of rabbits reduce the renal volume greatly and they have a relatively weak dilator action on the arteries of the periphery. These effects resemble those of adenylic acid more than those of histamine.

4. It is possible that the pressor-depressor substances of the blood are species-specific.

5. Human plasma, ascitic and spinal fluid which have been allowed to stand, yield extracts that are depressor and in their pharmacological action resemble closely extracts prepared from red blood cells and acetic acid extracts of tissues. Their constrictor effect on the kidneys is marked.

6. Histamine, choline, and adenosine depress the level of the blood pressure more effectively than they constrict the volume of the kidneys, while the reverse is true when adenylic acid is employed.

EXPERIMENTAL STUDIES ON ENCEPHALITIS

I. TRANSMISSION OF ST. LOUIS AND KANSAS CITY ENCEPHALITIS TO MICE*

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PLATE 2

(Received for publication, November 3, 1934)

An outbreak of encephalitis during August and September, 1933, in Missouri, centering in St. Louis where about 1,000 persons were affected, furnished an unusual opportunity for experimental studies on the nature of the disease. Attempts to transmit the encephalitis to laboratory animals were made promptly in St. Louis by Muckenfuss, Armstrong, and McCordock. Shortly after, the writers became interested in the possibility of transmitting the disease to a special breed of mice (1) which had proved highly susceptible to a virus associated with an encephalitis of sheep—louping ill (2). Consequently Dr. R. A. Muckenfuss was asked for sterile brain tissue from fatal cases in St. Louis and later a request for similar tissue from fatal cases in Kansas City was made to Dr. P. Stookey. They collected and generously sent us material, enabling inoculations to be begun September 1, 1933.

In November, 1933, Muckenfuss, Armstrong, and McCordock reported that a condition similar to the St. Louis disease had been transmitted to *Macacus rhesus* monkeys and maintained successfully for five passages (3). Following this, Webster and Fite reported that the encephalitis in both St. Louis and Kansas City was communicable by inoculation to mice, that the infectious agent was filtrable, highly virulent when instilled into the nasal passages of mice, and was neutralized by the serum of encephalitis convalescents from the 1933 epidemic but not by the serum of non-contacts (4). Further notes on the

* The writers gratefully acknowledge the aid of Dr. T. M. Rivers, both critical and suggestive, during the course of the work.

differentiation of the disease on an etiological basis (5, 6), on the presence of the disease in Paris, Illinois, in 1932 (5), and in New York in 1933 (5), and on the immunological properties of the virus (7) have been made by Webster and Fite; and reports of clinical and epidemiological data have been published by Hempelmann (8), Leake (9), and Bredeck (10). The aim of the present series of papers is to elaborate the preliminary reports from this laboratory and describe further experiments in detail.

Materials and Methods

The unusual feature in the technique of these experiments was the use of white-face, R. I. R., and Swiss mice. These specially bred animals have been described elsewhere in studies on inherited susceptibility and resistance to infectious diseases (1). They were reared in a breeding room in which environmental and dietary factors are uniform and in which no infectious diseases occurred. The mice had already proved highly susceptible to the neurotropic virus of sheep encephalitis, louping ill, developing a characteristic encephalitis (2) when inoculated either directly into the brain or into the nares. One strain of mice, the white-face, had proved equally susceptible to every microorganism and virus thus far tested,—*B. enteritidis*, *B. aertrycke*, *Pasteurella aviseptica*, *B. friedlaenderi*, pneumococcus, and louping ill virus. This, together with the Swiss strain, was therefore chosen for the present experiments.

Brain tissue from cortex, midbrain, and medulla was removed as soon as possible after the death of the patient, placed in 50 per cent glycerine and mailed to this laboratory where it was kept at $+4^{\circ}\text{C}$. until tested. For injection about 2 gm. of tissue from different areas was placed in a mortar, ground with sterile alundum for 10 minutes, taken up in 0.85 per cent salt solution to make a 10 per cent suspension, and tested for anaerobic and aerobic bacteria. Intracerebral injections into the mice were made with a 0.25 cc. tuberculin syringe and short No. 26 gauge needles. The mice were anesthetized lightly and given the inoculum into the brain through the skull, lateral to the mid-dorsal line and posterior to the eye. They were then placed one to five per cage and observed for 21 days. Signs of disease were looked for daily; prostrate or dead animals were examined at once or kept at $+4^{\circ}\text{C}$.

Transmission Experiments in Mice (Text-Fig. 1)

Specimen 1.—Brain tissue from a female patient aged 20 years who died at St. Louis City Isolation Hospital Aug. 21, 1933. Anatomical diagnosis: Encephalitis.¹ The first inoculation of tissue was made Sept. 1, 1933, into two white-

¹ We are indebted to Drs. Muckenfuss and McCordock for fixed tissue from, and histological diagnosis of the cases of encephalitis from St. Louis, and to Dr. Stookey for fixed tissue for microscopic study from the Kansas City cases.

face and two Swiss mice. All survived and were healthy after 21 days. A second inoculation was made Sept. 20 into two Swiss and two R. I. R. mice and a third inoculation Oct. 5 into four Swiss mice. All eight mice were living and well after 21 days.

Specimen 2.—Brain tissue from a female patient aged 67 years who died at St. Louis City Isolation Hospital Aug. 28, 1933. Anatomical diagnosis: Encephalitis. The first inoculation was made Sept. 1, 1933, into two white-face and two Swiss mice, a second inoculation Sept. 20 into two Swiss and two R. I. R. mice, and a third and fourth inoculation Oct. 5 and Oct. 19 into four Swiss and four white-face mice respectively. All sixteen mice remained well throughout the 21 day period of observation.

Specimen 3.—Brain tissue from a female patient who died at St. Louis City Isolation Hospital Aug. 25, 1933. Anatomical diagnosis: Encephalitis. The first inoculation of tissue was made Sept. 1, 1933, into two white-face and two Swiss mice. The mice remained well and active for 4 days. One white-face mouse was hyperesthetic and tremorous on the 5th day and dead on the 6th day. The second white-face mouse had convulsions on the 6th day and was killed. One Swiss mouse was dead on the 6th day; the other Swiss mouse remained healthy. Brains from the two white-face mice were removed aseptically, emulsified, suspended in saline, cultured, and diluted as before. Each suspension was then given intracerebrally to two white-face and two Swiss mice Sept. 9. After a 3 day healthy period, the mice developed hyperesthesia, tremors, convulsions, became prostrate and died on the 4th or 5th days. Brains from one of these white-face and one of these Swiss mice were removed, prepared and injected Sept. 14 each into white-face and Swiss mice. Mice receiving the brain emulsion from the white-face mouse died in 4, 4, 4, 4, 4, 4 days respectively, while those receiving the brain emulsion from the Swiss mouse died in 6, 7, 8, 8, 9, 6, 6, 6, 7, 8 days. Further passages of this Strain 3 at 4 to 7 day intervals to the number of about 50 have now been made. No changes in its behavior have been noted. A second inoculation from the original human material was made Sept. 11 into two white-face and two Swiss mice. The Swiss mice died with characteristic signs on the 7th day, the white-face mice on the 11th and 16th days. The brain from one of the Swiss mice was prepared and injected Sept. 20 into one Swiss and two R. I. R. mice. All died on the 5th day. A third attempt to transmit the disease from the human material was made Oct. 5. Four Swiss mice were injected but all remained healthy.

Specimen 4.—Brain tissue from a male patient aged 3 years who died at the Children's Hospital, St. Louis, Aug. 21, 1933. Anatomical diagnosis: Encephalitis. The first inoculation was made Sept. 1, 1933, into two white-face and two Swiss mice. One white-face mouse, A, was well until the 6th day at which time it developed convulsions and was killed. The second white-face mouse, B, was well until the 9th day when it was found to be prostrate and was killed. The two Swiss mice remained well during the 21 day period of observation. Brains of the two white-face mice were removed and emulsions prepared for the second

passage. The emulsion from the white-face Mouse A was injected into two white-face and two Swiss mice on Sept. 9; the brain emulsion from the other white-face Mouse B was injected similarly on Sept. 14. Mice receiving the A emulsion died on the 4th day. A brain from one of these white-face mice was emulsified and injected into two white-face and two Swiss mice on Sept. 14. These mice died on the 3rd, 3rd, 3rd, and 4th days following injection. A brain from one second passage Swiss mouse was treated similarly and injected into three white-face and three Swiss mice on Sept. 14. Two of the inoculated white-face mice died on the 8th day; the other survived. The three Swiss mice died on the 6th, 6th, and 7th days respectively. A brain emulsion of the first passage white-face Mouse B was given to two Swiss and two white-face mice on Sept. 14. They died on the 5th, 6th, 8th, and 8th days respectively. The brain from one Swiss mouse was given to two R. I. R. and two Swiss mice on Sept. 20. These third passage mice died with characteristic signs on the 5th, 6th, 5th, and 5th days respectively. The material was then stored in 50 per cent glycerine until Nov. 9, when a fourth passage was made. Fifth to tenth passages were then made with fresh tissue and the material was again stored in glycerine on Jan. 5, 1934. The results were consistent and similar in every way to those obtained with Strain 3. A second successful attempt to transmit the disease from the human material to mice was made Sept. 11, 1933, at which time two white-face and two Swiss mice were injected. One white-face mouse and one Swiss mouse died on the 6th day; the other two remained healthy 21 days. From the brain of the white-face mouse an emulsion was made and injected into two R. I. R. mice and 1 Swiss mouse. These died promptly on the 5th day with characteristic signs of encephalitis. A third attempt to transmit the disease from the human material was made on Oct. 5 when four Swiss mice were injected, and a fourth attempt on Oct. 19 when four white-face mice were injected. All remained alive and well 21 days.

Specimen 5.—Brain tissue from a male patient who died at Deaconess Hospital, St. Louis, Sept. 1, 1933. Anatomical diagnosis: Encephalitis. The first inoculation was made September 6, 1933, into two white-face and two Swiss mice. One Swiss mouse was found prostrate and was sacrificed on the 8th day; the others remained well. A second passage was made from the brain of the positive mouse into two Swiss mice and one R. I. R. mouse on Sept. 20. They died on the 5th, 5th, and 6th days respectively. An emulsion for third passage was made from the brain of one of the Swiss mice and injected into four Swiss and four R. I. R. mice on Sept. 27. They died on the 5th, 5th, 5th, 6th, 4th, 5th, 5th, and 6th days respectively with typical signs of encephalitis. The material was then stored in glycerine until Nov. 9 when a fourth passage was made. Fifth to ninth passages were then made in rapid succession and the ninth passage material was again stored in glycerine. The results of these passages were similar in every way to previous tests with this Strain 5 and with Strains 3 and 4. Three additional unsuccessful attempts to transmit the disease from the human material to mice were made on Oct. 5 at which time four Swiss mice were injected, on Oct. 19

four white-face mice, and on Nov. 23 four Swiss mice. All animals remained well during the 21 day period of observation.

Specimen 6.—Brain tissue from a female patient aged 63 who died at St. Louis City Isolation Hospital Sept. 2, 1933. Anatomical diagnosis: Encephalitis. Four unsuccessful attempts were made to get positive results in mice with this material. On Sept. 6, 1933, two white-face and two Swiss were injected; on Sept. 20 two Swiss and two R. I. R.; on Oct. 5 four Swiss; and on Oct. 19 four white-face mice. All survived and were well during the period of observation.

Specimen 7.—Brain tissue from a female patient aged 75 who died at St. Louis County Hospital Aug. 29, 1933. Anatomical diagnosis: Encephalitis. Four unsuccessful attempts were made with this specimen at the same time and in the same manner as in the case of Specimen 6. All sixteen injected mice remained well.

Specimen 8.—Brain tissue from a male patient aged 73 who died at De Paul Hospital, St. Louis, Aug. 30, 1933. Anatomical diagnosis: Encephalitis. The first inoculation was made Sept. 8, 1933, into four white-face and four Swiss mice. One white-face mouse died on the 9th day and one Swiss mouse on the 11th day. The six remaining mice continued well throughout the 21 day period of observation. The brain from the Swiss mouse was prepared and injected into two R. I. R. and two Swiss mice. These second passage animals died on the 5th day. Brains from two of these animals were placed in glycerine and not tested until Nov. 9 and again on Nov. 15. The five injected animals remained well and no further attempts to recover the virus from the second passage material were made. The original human material was tested again, on Oct. 5, Oct. 19, and Nov. 23 by injecting four Swiss, four white-face, and four Swiss mice respectively. All remained well during the 21 day period.

Specimen 9.—Brain tissue from a female patient aged 70 who died at the Jewish Hospital, Kansas City, Sept. 25, 1933. Anatomical diagnosis: Encephalitis. Four unsuccessful attempts were made with this material to transmit the disease to mice. On Sept. 28, 1933, four Swiss mice were inoculated; on Oct. 6 four Swiss mice; on Oct. 19 four white-face mice; on Nov. 13 four white-face mice. All remained well and were discarded 18 days after the injection was made.

Specimen 11.—Brain tissue from a male patient aged 70 who died at the General Hospital, Kansas City, Oct. 5, 1933. Clinical diagnosis: Encephalitis.² The first transmission experiment was made Oct. 9, 1933, by injecting four Swiss mice. All remained healthy during the 21 day period of observation. A second attempt was made with the human tissue on Oct. 19 by injecting four white-face mice. One with convulsions and later prostrate on the 8th day was killed and its brain prepared and injected Oct. 29 into two white-face and two Swiss mice. The other three white-face mice remained well. The second passage mice were all

²No record of histological study of brain tissue from this case has been located.

dead on the 3rd day. The brains from one white-face and one Swiss mouse were pooled and injected Nov. 4 into three Swiss mice. They were all dead on the 4th day. A fourth and fifth passage were made in Swiss mice Nov. 9 and Nov. 15. All died in 3 to 5 days. Brains of the fifth passage mice were preserved in glycerine until Dec. 16 when the sixth to ninth passages were made. This strain from Kansas City behaved in every way like the St. Louis strains and gave rise in the injected mice to the typical signs of encephalitis. A third attempt with the original human material was made Nov. 3. Four white-face mice were injected and one was prostrate and one was dead on the 6th day. The prostrate mouse was sacrificed, its brain removed and injected Nov. 10 into three Swiss mice. These were all dead on the 5th day. Their brains were removed and stored in glycerine.

Specimen 12.—Brain tissue from a female patient aged 68 who died at the De Paul Hospital, St. Louis, Sept. 3, 1933. Anatomical diagnosis: Encephalitis. One transmission experiment was made Oct. 16, 1933, by inoculating four white-face mice. The mice remained well.

Specimen 13.—Brain tissue from female patient who died at the Jewish Hospital, St. Louis, Sept. 3, 1933. Anatomical diagnosis: Encephalitis. One attempt to transmit the disease was made with this specimen on Oct. 16, 1933. Four white-face mice were injected but none showed any sign of disease for the 21 days following.

Specimen 14.—Brain tissue from male patient aged 65 who died at St. Louis County Hospital Sept. 6, 1933. Anatomical diagnosis: Encephalitis. One injection of the brain tissue was made on Oct. 16, 1933, into four white-face mice. All remained well.

Specimen 15.—Brain tissue from male patient aged 80 years who died at the St. Louis County Hospital Sept. 10, 1933. Anatomical diagnosis: Encephalitis. One injection of tissue into four white-face mice was made Oct. 16, 1933, with negative results.

Specimen 16.—Brain tissue from a female patient aged 75 who died at Barnes Hospital, St. Louis, Sept. 14, 1933. Anatomical diagnosis: Encephalitis. An injection of this material was made Oct. 16, 1933, into four white-face mice. Three remained well, but one, with tremors and convulsions on the 8th day, was sacrificed. Its brain, prepared and injected Oct. 25 into two white-face and two Swiss mice, was fatal to all four on the 4th day. Brains from these mice were pooled and a third passage made Oct. 30 into two white-face and two Swiss mice. All were in convulsions or prostrate on the 4th day. A fourth passage, Nov. 4, fifth passage, Nov. 8, and sixth passage, Nov. 15, were made successfully and the material preserved in glycerine. This strain behaved in every way like the others.

Specimen 17.—Brain tissue from male patient aged 65 years who died at the Jewish Hospital, St. Louis, Sept. 4, 1933. Anatomical diagnosis: Encephalitis. One injection of this material was made Oct. 16, 1933, into four white-face mice. All remained well during the 21 day period of observation.

Specimen 18.—Brain tissue from male patient aged 23 years who died at the Deaconess Hospital, Sept. 13, 1933. Anatomical diagnosis: Encephalitis. One attempt to transmit the disease with this specimen was made Oct. 16, 1933, in four white-face mice. The result was negative.

Specimen 19.—Brain tissue from male patient who died at the De Paul Hospital, St. Louis, on or about Sept. 15, 1933. Anatomical diagnosis: Encephalitis. One injection of this material Oct. 16, 1933, into four white-face mice was without effect.

Specimen 20.—Brain tissue from male patient aged 74 who died at St. Margaret's Hospital, Kansas City, Oct. 11, 1933. Anatomical diagnosis: Encephalitis. Two attempts were made to transmit the disease with this material, one on Oct. 14, 1933, when four Swiss mice were injected, and one on Oct. 19 when four white-face mice were injected. All of the animals remained healthy throughout the 3 week period of observation.

The experiments showed that brain tissue from clinical cases of encephalitis, with histological lesions characteristic of the disease, and free of bacteria, when injected intracerebrally into mice of special strains, gave rise in them to a characteristic picture of encephalitis. After an incubation period of 5 to 10 days, hyperesthesia and tremors were noted, progressing to convulsions, prostration, and death in 6 to 12 days. This encephalitis was reproduced in mice in series by injecting emulsions of bacteriologically sterile brain from the sick mice into the brains of normal mice (Text-fig. 1). No change in the clinical disease took place on passage except that the incubation period was soon shortened to 3 to 4 days and the duration of life to 4 to 6 days. Five of eleven specimens tested within 14 days of the death of the patient were positive—45 per cent (Table I). Retests of these were positive until the 29th day but were negative thereafter. One of three specimens was positive when first tested 32 to 36 days after the death of the patient; five not tested until the 36th to 43rd day were negative. Apparently the encephalitis-producing activity of the human tissue when preserved in glycerine is limited to about 32 days (Table I). Finally, the five active specimens from St. Louis cases and the one from Kansas City were identical in so far as could be determined in their effects on the mice.

Pathology of Encephalitis in Mice

Lesions in mice prostrate or dying of the disease were apparently limited to the central nervous system. Mononuclear leucocytes were

dead on the 3rd day. The brains from one white-face and one Swiss mouse were pooled and injected Nov. 4 into three Swiss mice. They were all dead on the 4th day. A fourth and fifth passage were made in Swiss mice Nov. 9 and Nov. 15. All died in 3 to 5 days. Brains of the fifth passage mice were preserved in glycerine until Dec. 16 when the sixth to ninth passages were made. This strain from Kansas City behaved in every way like the St. Louis strains and gave rise in the injected mice to the typical signs of encephalitis. A third attempt with the original human material was made Nov. 3. Four white-face mice were injected and one was prostrate and one was dead on the 6th day. The prostrate mouse was sacrificed, its brain removed and injected Nov. 10 into three Swiss mice. These were all dead on the 5th day. Their brains were removed and stored in glycerine.

Specimen 12.—Brain tissue from a female patient aged 68 who died at the De Paul Hospital, St. Louis, Sept. 3, 1933. Anatomical diagnosis: Encephalitis. One transmission experiment was made Oct. 16, 1933, by inoculating four white-face mice. The mice remained well.

Specimen 13.—Brain tissue from female patient who died at the Jewish Hospital, St. Louis, Sept. 3, 1933. Anatomical diagnosis: Encephalitis. One attempt to transmit the disease was made with this specimen on Oct. 16, 1933. Four white-face mice were injected but none showed any sign of disease for the 21 days following.

Specimen 14.—Brain tissue from male patient aged 65 who died at St. Louis County Hospital Sept. 6, 1933. Anatomical diagnosis: Encephalitis. One injection of the brain tissue was made on Oct. 16, 1933, into four white-face mice. All remained well.

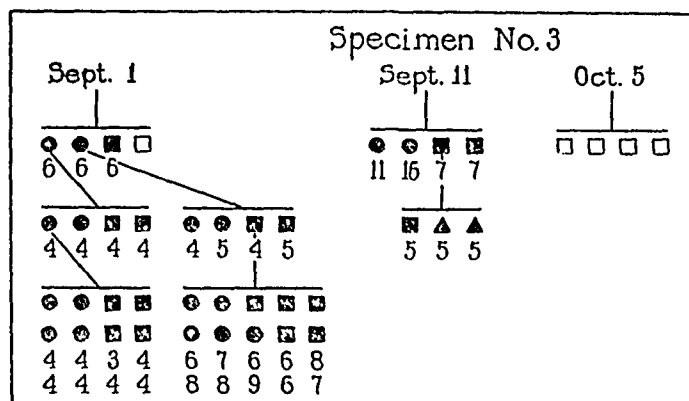
Specimen 15.—Brain tissue from male patient aged 80 years who died at the St. Louis County Hospital Sept. 10, 1933. Anatomical diagnosis: Encephalitis. One injection of tissue into four white-face mice was made Oct. 16, 1933, with negative results.

Specimen 16.—Brain tissue from a female patient aged 75 who died at Barnes Hospital, St. Louis, Sept. 14, 1933. Anatomical diagnosis: Encephalitis. An injection of this material was made Oct. 16, 1933, into four white-face mice. Three remained well, but one, with tremors and convulsions on the 8th day, was sacrificed. Its brain, prepared and injected Oct. 25 into two white-face and two Swiss mice, was fatal to all four on the 4th day. Brains from these mice were pooled and a third passage made Oct. 30 into two white-face and two Swiss mice. All were in convulsions or prostrate on the 4th day. A fourth passage, Nov. 4, fifth passage, Nov. 8, and sixth passage, Nov. 15, were made successfully and the material preserved in glycerine. This strain behaved in every way like the others.

Specimen 17.—Brain tissue from male patient aged 65 years who died at the Jewish Hospital, St. Louis, Sept. 4, 1933. Anatomical diagnosis: Encephalitis. One injection of this material was made Oct. 16, 1933, into four white-face mice. All remained well during the 21 day period of observation.

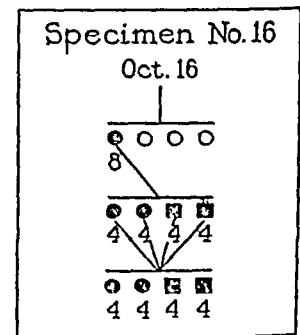
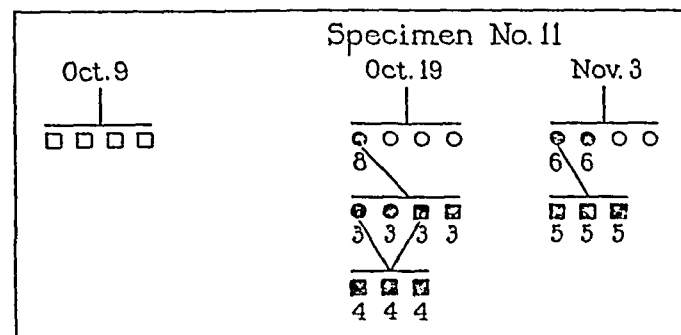
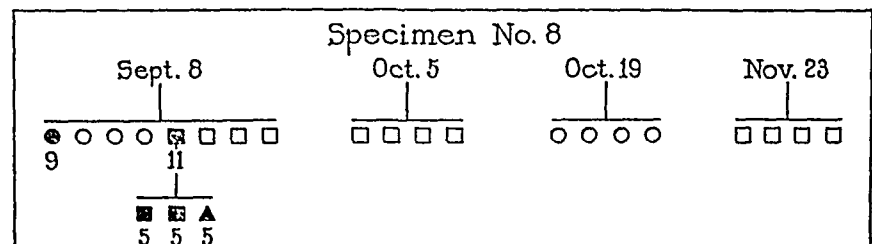
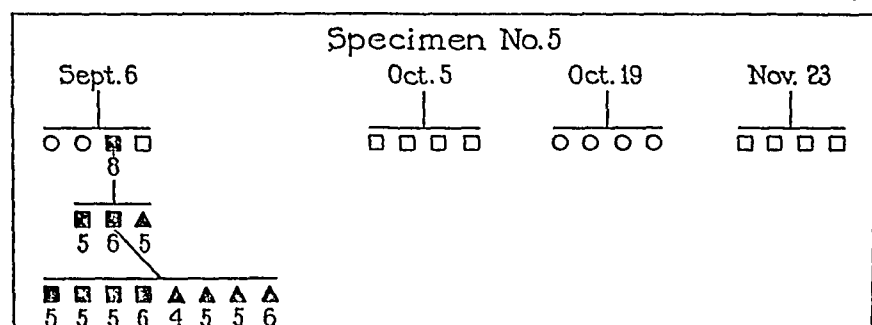
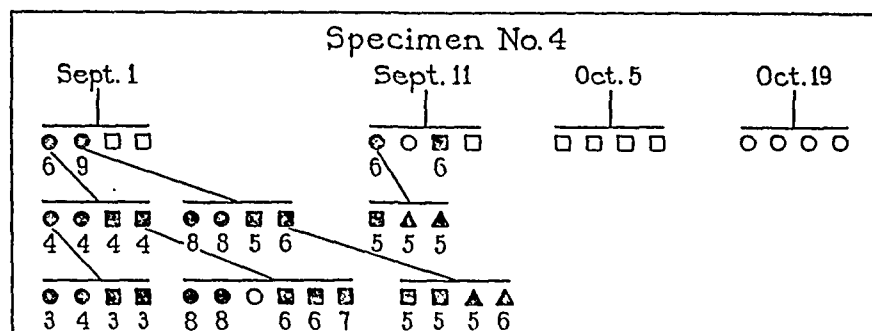
TABLE I
Survival of Virus in Human Brain Tissue from Fatal Cases of Encephalitis (St. Louis and Kansas City)

Specimen No.	Date of death of patient	Date of inoculation of tissue	Interval between patient's death and inoculation of tissue	Results of inoculation
	1933	1933	days	
1	Aug. 21	Sept. 1	11	0
		" 20	30	0
2	" 28	Oct. 5	45	0
		Sept. 1	4	0
		" 20	23	0
		Oct. 5	38	0
3	" 25	" 19	52	0
		Sept. 1	7	+
		" 11	17	+
4	" 21	Oct. 5	44	0
		Sept. 1	11	+
		" 11	21	+
		Oct. 5	45	0
5	Sept. 1	" 19	59	0
		Sept. 6	5	+
		Oct. 5	29	0
		" 19	43	0
6	" 2	Nov. 23	77	0
		Sept. 6	4	0
		" 20	18	0
		Oct. 5	33	0
7	Aug. 29	" 19	47	0
		Sept. 6	8	0
		" 20	22	0
		Oct. 5	37	0
8	" 30	" 19	51	+
		Sept. 8	9	0
		Oct. 5	27	0
		" 19	41	0
9	Sept. 25	Nov. 23	76	0
		Sept. 28	3	0
		Oct. 6	11	0
		" 19	24	0
11	Oct. 5	Nov. 3	39	0
		Oct. 9	4	0
		" 19	14	+
12	Sept. 3	Nov. 3	29	+
13	" 3	Oct. 16	43	0
14	" 6	" 16	43	0
15	" 10	" 16	40	0
16	" 14	" 16	36	0
17	" 4	" 16	32	+
18	" 13	" 16	42	0
19	" 14	" 16	33	0
20	Oct. 11	" 16	32	0
		" 14	3	0
		" 19	8	0



○ = White-face
 □ = Swiss
 △ = R.I.R.

White symbols = survived
 Black " = died
 Numbers = Survival in days after injection



TEXT-FIG. 1. Successful transmission and maintenance of encephalitis (St. Louis and Kansas City) in mice.

Transmission of the St. Louis encephalitis to *Macacus rhesus* monkeys for five passages by presumably bacteria-free suspensions of brain tissue from fatal human cases (3) is suggestive that this disease is a virus infection, but unfortunately the clinical disease of the passage monkeys becomes progressively less definite and finally disappears, even though a technique of massive inoculation is employed. It is of special interest, therefore, that the St. Louis and Kansas City diseases can be transmitted to mice in indefinite series by small injections of bacteria-free brain emulsions from fatal cases, and that a characteristic and consistent clinical and pathological picture results. From these experiments, it is inferred that at least one type of encephalitis is infectious in nature and incited by a virus agent.

CONCLUSIONS

1. Mice of special strains injected intracerebrally with a 10 per cent emulsion of bacteria-free brain tissue from fatal cases of encephalitis in St. Louis and Kansas City develop a characteristic and fatal encephalitis.
2. Transmission of the disease can be continued indefinitely by injecting the bacteria-free brain tissue from the infected mice into healthy mice.
3. In the injected mice there is a 3 to 4 day incubation period, followed by hyperesthesia, coarse tremors, convulsions, prostration, and death in from 4 to 6 days.
4. The lesions in the mice with experimental encephalitis consist chiefly of perivascular accumulations of mononuclear leucocytes throughout the brain, stem, cord, and the pia, and destruction of pyramidal cells in the lobus piriformis and cornu Ammonis.
5. The human encephalitis brain tissue preserved in glycerine from the time of death of the patient apparently loses its infectivity for mice in about 32 days.

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collected about many small blood vessels throughout the brain, stem, and cord, especially near the meninges (Figs. 1 and 2). The cells were likewise present in the pia in the perivascular and neighboring spaces. Near them, glial cells were sometimes mobilized. Most striking, however, were the lesions in the lobus piriformis and cornu Ammonis of the cortex (Figs. 1, 3, and 4). Here the bands of pyramidal cells, normally very regular in their arrangement, showed areas of sudden widening, distortion, and nearly complete destruction. The pyramidal cells contiguous to the normal area were scattered, some with large ameboid outlines, others with pycnotic nuclei and shrunken cytoplasm. Glial cells and scattered mononuclear cells were conspicuous. Adjacent to this early lesion there were often areas in which no pyramidal cells remained, consisting of either a relatively homogeneous necrotic substance with an occasional shrunken glial cell, or leucocyte, or areas of definite thinning and softening. Thus far, no specific cellular pathology has been noted beyond certain intranuclear bodies in the choroid and glial cells of certain mice, which are being subjected to further study.

DISCUSSION

Primary encephalitis breaks out occasionally in epidemic form. For example, lethargic or epidemic encephalitis did so following the influenza pandemic of 1917-18 in Europe and the United States; Japanese Type B encephalitis occurred during August and September, 1924 and 1933; and the encephalitis now under study was prevalent in St. Louis in August and September, 1933. Some infectious agent has been postulated as responsible for these epidemics but searches for the agent in the case of epidemic and Japanese Type B encephalitis, while eliminating the more common bacterial species and suggesting the presence of a virus, have not yet disclosed the presence of any specific agent. Still less clear is our understanding of the nature of the sporadic cases of primary encephalitis, which differ so widely in clinical and time and space patterns. The main problem is therefore whether the types of primary encephalitis referred to are one or many diseases and whether they, like certain types of encephalitis of animals, are to be regarded as infections, each one associated with a specific filtrable virus.

Transmission of the St. Louis encephalitis to *Macacus rhesus* monkeys for five passages by presumably bacteria-free suspensions of brain tissue from fatal human cases (3) is suggestive that this disease is a virus infection, but unfortunately the clinical disease of the passage monkeys becomes progressively less definite and finally disappears, even though a technique of massive inoculation is employed. It is of special interest, therefore, that the St. Louis and Kansas City diseases can be transmitted to mice in indefinite series by small injections of bacteria-free brain emulsions from fatal cases, and that a characteristic and consistent clinical and pathological picture results. From these experiments, it is inferred that at least one type of encephalitis is infectious in nature and incited by a virus agent.

CONCLUSIONS

1. Mice of special strains injected intracerebrally with a 10 per cent emulsion of bacteria-free brain tissue from fatal cases of encephalitis in St. Louis and Kansas City develop a characteristic and fatal encephalitis.

2. Transmission of the disease can be continued indefinitely by injecting the bacteria-free brain tissue from the infected mice into healthy mice.

3. In the injected mice there is a 3 to 4 day incubation period, followed by hyperesthesia, coarse tremors, convulsions, prostration, and death in from 4 to 6 days.

4. The lesions in the mice with experimental encephalitis consist chiefly of perivascular accumulations of mononuclear leucocytes throughout the brain, stem, cord, and the pia, and destruction of pyramidal cells in the lobus piriformis and cornu Ammonis.

5. The human encephalitis brain tissue preserved in glycerine from the time of death of the patient apparently loses its infectivity for mice in about 32 days.

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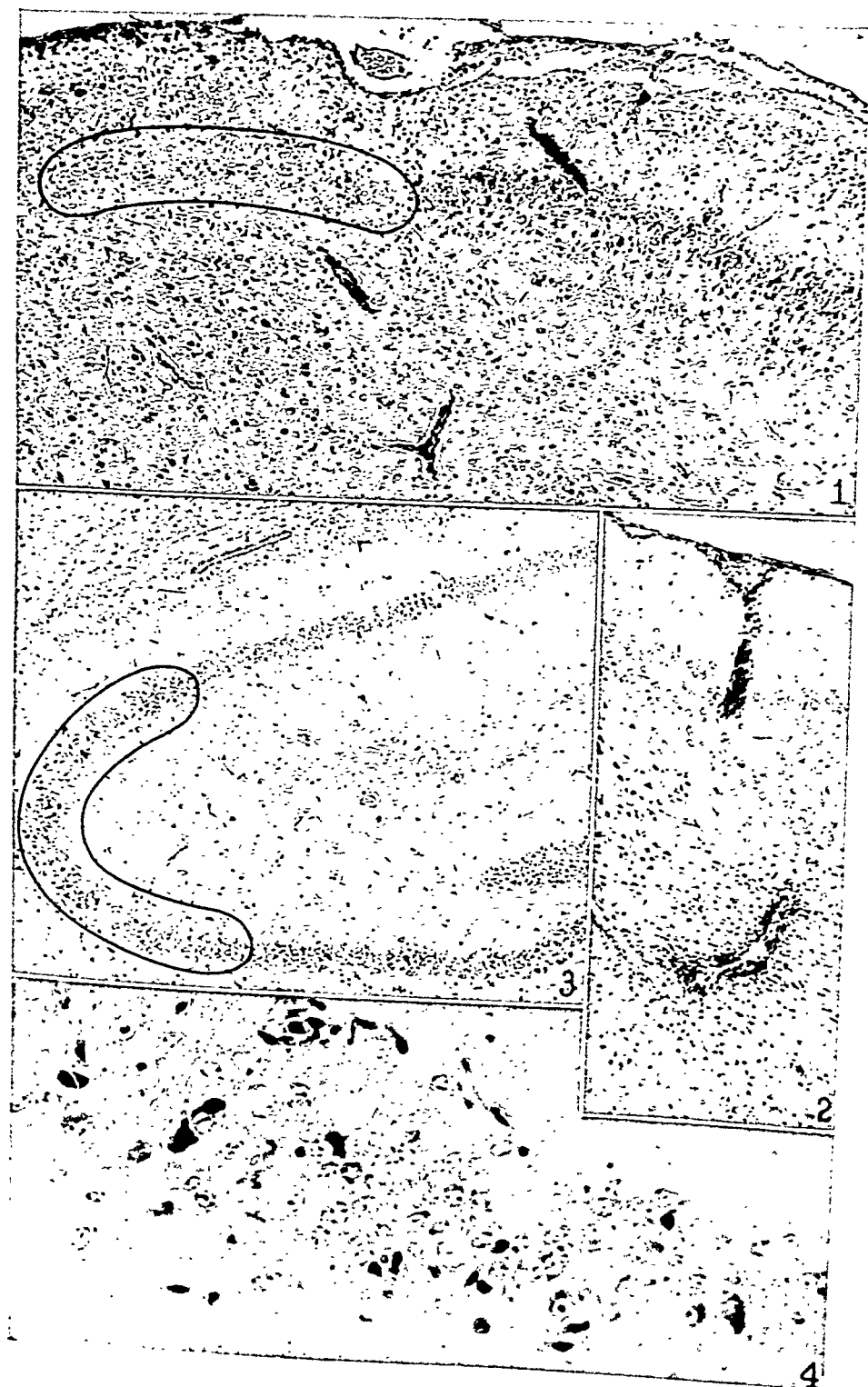
EXPLANATION OF PLATE 2

FIG. 1. Lobus piriformis. Perivascular and subpial accumulations of mononuclear cells and destruction of pyramidal cells (outlined area), with scattered glial cells and leucocytes. Hematoxylin and eosin stain. $\times 100$.

FIG. 2. Lobus piriformis. Perivascular, mononuclear leucocyte infiltration. Hematoxylin and eosin stain. $\times 100$.

FIG. 3. Cornu Ammonis. Specific destruction of pyramidal cells (outlined area), with little or no surrounding reaction. Hematoxylin and eosin stain. $\times 100$.

FIG. 4. Cornu Ammonis. Destruction of pyramidal cells. Normal cells (right); ameboid and pycnotic cells (center and left); cell destruction (left). Hematoxylin and eosin stain. $\times 420$.



I. BARTONELLA INCIDENCE IN SPLENECTOMIZED BILE FISTULA DOGS

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PLATE 3

(Received for publication, October 16, 1934)

This paper deals with the factors responsible for the pigmentary disturbances previously described (8) in the splenectomized bile fistula dog. In the absence of demonstrable infection in such animals it had been assumed that the periods of bile pigment surplus and anemia were of physiological origin related to a lack of spleen and bile constituents. The observations given below point to "*Bartonella canis*" as the probable cause. The bile fistula dog alone shows no such changes, and the splenectomized dogs in our kennels have not spontaneously developed an anemia. Dogs having a combination of splenectomy and a bile fistula, sooner or later develop the picture of anemia and pigment excess described below. This condition was first described by Hooper and Whipple (2), working with open bile fistula dogs subsequently splenectomized. These dogs always showed moderate infection of the bile fistula tract. The use of the closed sterile bile fistula (10) and the gall bladder-renal fistula (3) did away with bacterial infection of the biliary tree, yet such dogs when splenectomized showed the same periods of anemia and excess pigment output.

Other investigators have observed periods of anemia in splenectomized dogs. Krumbhaar (6) holds an initial anemia period to be a physiologic response to the loss of the spleen. Kikuth (4) and others (9) have demonstrated cyclical periods of anemia following splenectomy. They have shown the relationship of such periods to the presence in and on the red blood cells of bodies which they consider to belong to the *Bartonella* group, which they have called *Bartonella canis*. Kikuth has reported two types of the disease in dogs, an acute form which runs a comparatively short course and ends with death, and a chronic form which

persists over a long period of time. He has observed that the characteristic bodies appear in the blood in greatest numbers just before the greatest drops in the red count and hemoglobin occur. He has been able to reproduce the condition in other splenectomized dogs by inoculation of blood from infected animals. His attempts to cultivate the bodies were unsuccessful.

To determine whether *Bartonella canis* is associated with the pigment and blood phenomena observed in splenectomized bile fistula dogs, the following experiments have been carried out.¹

Methods

Blood smears from gall bladder-renal and sterile closed fistula dogs as well as normal and splenectomized dogs were made at frequent intervals. The fistula dogs were among those being used for pigment studies, and were kept under standard conditions as to diet, bile intake, and routine care prior to and during the course of the investigation. Paper II (5) of this series relates full details as to the care and details of study of the pigment metabolism of these dogs.

The blood smears were stained with Wright's stain by the usual method, and were examined under oil immersion for *Bartonella* bodies.

Frequent red cell counts were made in conjunction with hemoglobin and hematocrit estimations.

In transmission experiments, the necks of the dogs were shaved and cleansed using soap, alcohol, and iodine technique. The blood (5-10 cc.) was removed from the jugular vein of the donor in a sterile syringe, and introduced, without the addition of an anticoagulant, into the jugular vein of the recipient.

EXPERIMENTAL OBSERVATIONS

Four splenectomized gall bladder-renal fistula dogs were studied. Three of these were showing typical severe disturbances in pigment metabolism. The fourth (32-74) had never showed the marked degree of blood destruction that was observed in other animals. This dog will be described in some detail later. In the three former animals, bodies have been found associated with the red blood cells which are morphologically indistinguishable from *Bartonella canis*, according to descriptions and photographs of Kikuth (4) and Regendanz and Reichenow (9).

Fig. 1 shows the characteristic appearance of the bodies in the blood of a dog (29-329) in which they are quite numerous. They

¹ We are grateful to Dr. Cornelius P. Rhoads for suggesting the possible association of *Bartonella canis* with the phenomena observed in these animals.

appear as dark blue staining elongated beaded rod-like forms, and most frequently lie in a radial direction, on or in the cell. Pale vacuolated spaces in the affected red cell are commonly noted at one or both ends of the bodies. In addition to the beaded rod-like forms, of which 1-4 have been observed in association with one cell, there are short rods and small coccoid bodies, which may be present in the

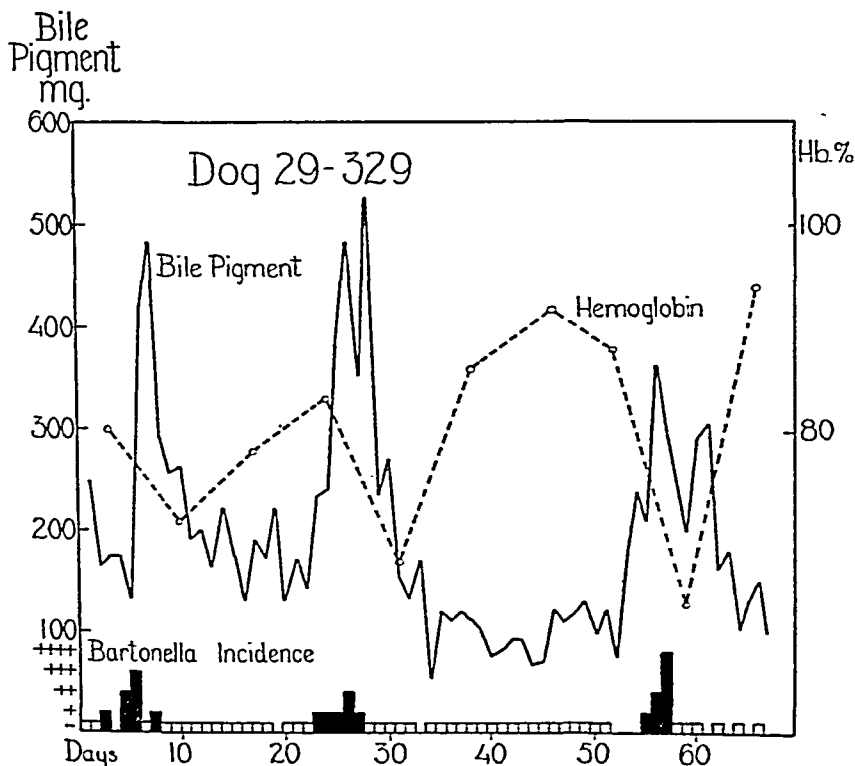


CHART A. *Bartonella* incidence in splenectomized bile fistula dogs.

same cells with the rods, or many of this type alone may be numerous in other cells. Tiny ring forms such as those described by Regendanz and Reichenow are frequently present, and are most numerous early in the course of a typical period of blood destruction. Occasional narrow semilunar sickle-like forms have been observed. Such bodies have a granular appearance and sometimes extend, at the periphery of the cell, about two-thirds of the way around it.

Usually the more severe the involvement of the animal, as measured by the blood destruction, the more diverse are the types of bodies present. A short time after the initial appearance of such bodies in the blood, usually 3-5 days, every dog which has been observed has shown evidences of blood destruction. This reflects itself in a drop in the hemoglobin percentage, and in a rise in the bile pigment output. The bodies disappear quite abruptly from the blood,

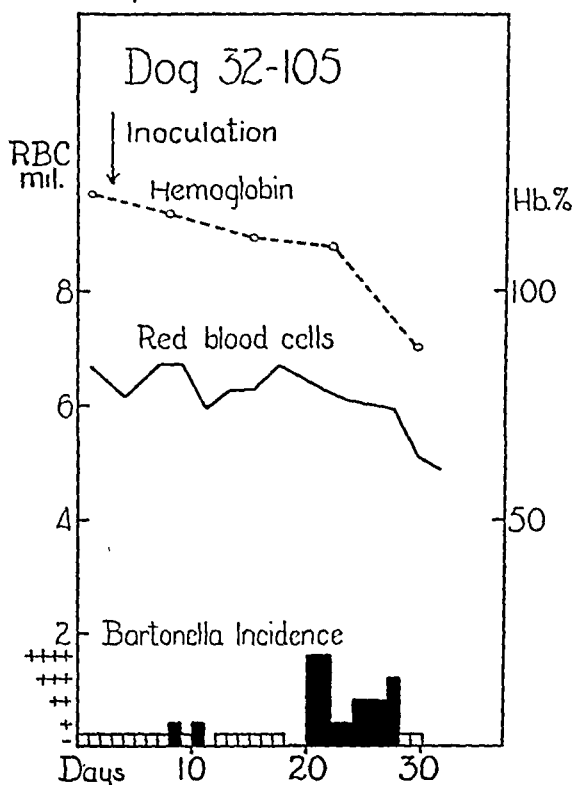


CHART B. *Bartonella* incidence and anemia in splenectomized dog.

and intervals of a few days to a month may elapse before they reappear. Not one of the dogs has so far spontaneously recovered from the involvement.

Chart A shows an interval of about 2 months during the course of one of the splenectomized gall bladder-renal fistula dogs (29-329). It represents the daily bile pigment output, the weekly hemoglobin determination, and frequent examinations of blood smears. The periodic nature of the changes in these determinations

is readily seen. A few days before the rise of the bile pigments with the associated hemoglobin drop, characteristic bodies appeared in the blood, and reached their greatest numbers a day or two before the highest rise in bile pigment. A rough quantitative estimation of the bodies is designated by plus marks. One plus indicates infrequent bodies, less than one per oil immersion field; two plus, one or two cells containing bodies per oil immersion field; three plus, more than two; and four plus numerous bodies in every field. Fig. 1 would indicate a four plus reaction. Negative smears are shown by hollow blocks.

Such periods are characteristic of those observed in other animals. In one dog (31-359), never more than one plus smears have been observed except on a single occasion, when the smear showed numerous bodies. In another dog (32-74) a single one plus smear has been seen.

This dog (32-74) was operated upon under ether January 10, 1933. No pigment disturbances were noted until June 11, at which time a very slight period of pigment excess started. This lasted for 9 days. From this time, the pigments remained within control limits. On August 14, 1933, it was inoculated with blood from Dog 31-359, while the latter animal was at the height of a breakdown period. Two days after the inoculation, the bile pigment elimination increased, and there was a drop in the hemoglobin. From this time the dog continued to have regular, but slight periods of pigment overproduction. One smear out of many studied during this interval was found to be positive. A second inoculation of "infected" blood has not caused positive smears, although the animal has continued to have infrequent slight periods of pigment overproduction.

Transmission Experiments

Two splenectomized dogs (33-292, 32-105) were selected for transmission experiments.

One of these animals (32-105) had been operated upon 6 months previous to the inoculation, and its blood picture had been followed. At no time had there been evidence of an anemia. Blood smears were examined daily for a period of 28 days preceding the inoculation, and none showed *Bartonella* bodies. Chart B shows the hemoglobin, erythrocyte count, and result of blood smear examinations for a month subsequent to the intravenous injection into this dog of 10 cc. of blood from a splenectomized biliary-renal fistula dog (29-329), which on the day of inoculation showed a one plus blood smear. For 5 days subsequent to the inoculation there was no significant change in the erythrocyte count or in the hemoglobin determination. On the 6th day, the smear was positive for *Bartonella* bodies, and 2 days later was again positive. There was, at that time, a slight drop in the red cell count, and in the hemoglobin percentage. Nine days later, numbers of bodies appeared in the blood, and persisted for 8 days, following which there was a moderate, but definite drop in the hemoglobin and in the red count.

The course of the second splenectomized dog (33-292) is illustrated in Chart C. This dog had had a splenectomy 9 days previous to the inoculation. Blood smears had been studied daily for 28 days previous to the inoculation, and all had been negative. Ten cc. of blood from the same dog (29-329) were injected intravenously on the same day that the previous splenectomized animal was inoculated. Two days after inoculation, bodies were first noted in the blood, and the smears continued to be positive for 15 days. This was associated with a definite drop

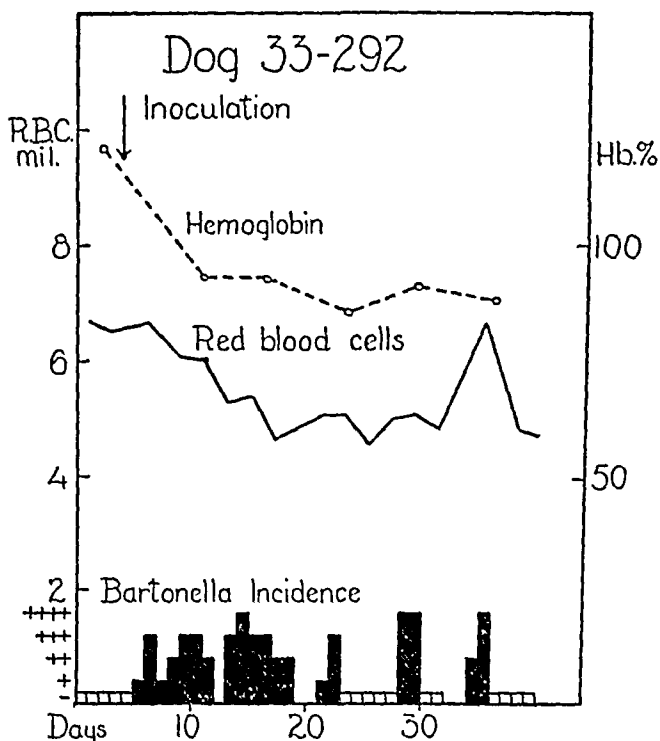


CHART C. *Bartonella* incidence and anemia in splenectomized dog.

in the number of circulating red cells, and in the hemoglobin. The cyclical tendency after the initial positive period shows up to advantage in this chart.

The third animal to be inoculated was a splenectomized closed sterile fistula dog (33-51) which had been operated upon 9 days previously. Smears studied previous to the inoculation were negative. Four days after inoculation a four plus smear was observed, and the bile pigments showed a definite rise. This animal continued to have cyclical periods of blood destruction.

CONTROL EXPERIMENTS

Daily blood smears from a normal dog (31-271) were examined for 9 consecutive days. All were negative. On the 9th day the dog was inoculated with 10 cc. of

blood from a splenectomized gall bladder-renal fistula dog (29-329), which showed at the time a one plus smear. For a period of 31 days, daily smears were examined. Red blood counts were made tri-weekly, and hemoglobin and hematocrit estimations were carried out weekly. At no time was there any significant variation in the latter three determinations, and no *Bartonella* bodies could be found in any of the blood smears.

In addition, daily blood smears from four other normal dogs have been studied over a period of time, and no *Bartonella* bodies have been noted.

Cultural Attempts

So far, attempts to culture the bodies have been futile. The effort has not been undertaken on a large scale, but blood from two dogs showing characteristic bodies was introduced in various dilutions into leptospira media prepared according to the method of Noguchi (7). The cultures were incubated at 30°C. and were examined at intervals up to a month. A few tubes were contaminated with moulds, otherwise no growth was observed.

Therapeutic Measures

Both Kikuth and Regendanz and Reichenow have reported "sterilization" of the blood stream of animals bearing *Bartonella canis* by the use of neosalvarsan. We have carried out one preliminary test along these lines. A dog (29-329) which has shown regular periodic intervals of blood destruction associated with the presence of *Bartonella* bodies over a period of 8 months was given a single intravenous dose of neosalvarsan. 210 mg. or the equivalent of 15 mg. per kilo weight were injected on a day when the blood smear was three plus. The following day the smear was negative, and over a period of 7 weeks, frequent examinations have remained negative. In the meantime, no demonstrable periods of blood destruction have occurred; the hemoglobin has risen almost to the control level; and the bile pigments have remained low.

Before the association of *Bartonella canis* to the condition was recognized, spleen extracts were fed to a dog on the assumption that the periods of blood destruction might be related to the lack of some intrinsic spleen factor. Not until after the spleen feeding had been discontinued, was the relation of *Bartonella canis* demonstrated. At this time the animal was again showing typical cyclical periods of

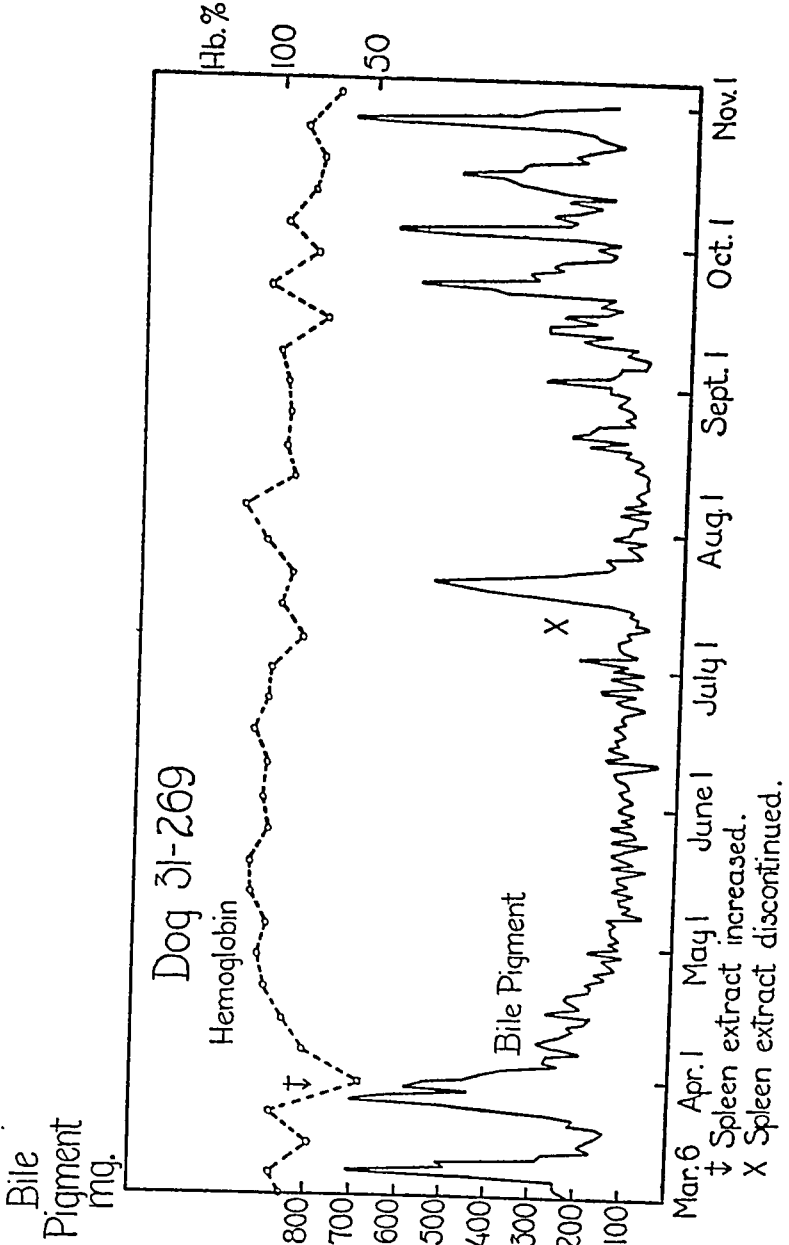


CHART D. Spleen extract feeding in splenectomized bile fistula dog.

anemia and pigment overproduction. *Bartonella* bodies were demonstrated in large numbers, in relation to the anemic periods. Since the results obtained were so clear-cut it seems worth while to include them in this report.

A dog (31-269) was splenectomized and a gall bladder-renal fistula made on March 28, 1932, with an uneventful recovery. It remained normal, as far as pigment metabolism was concerned, until September 22, when the first period of excess elimination of bile pigment began. During the following 3 months there were six distinct periods of bile pigment excess and anemia. At the height of the seventh cycle (December 25, 1932) spleen extracts were added to the diet in the following amounts: 3.6 gm. of No. 55 and 5 gm. of No. 343.² Cycles of pigment overproduction continued so that on January 18, 1933, 24 days later, the amounts of extract were doubled. Thirteen days later, 11.25 gm. of liver extract No. 55 were added in addition to the spleen factor, and the dog was kept on this diet for 3 months. The cycles still persisted, so the No. 55 spleen extract was increased to 10.8 gm. and the No. 343 spleen extract to 15 gm. The liver extract was discontinued at this time. The above amounts of spleen extract are equivalent to about 700 gm. of fresh spleen.

Chart D shows what occurred after the extracts were increased to the above stated amounts. The periods of pigment overproduction are seen to stop abruptly, and the amounts of bile pigment eliminated thereafter are comparable to the basal control level. The hemoglobin rises and remains at about 100 per cent. On July 13, 1933, 2½ months later, spleen feeding was discontinued, and 4 days later a typical period of pigment excess occurred. Similar periods, increasing in severity, followed rapidly over an interval of 5 months until December 12, 1933, when the dog was accidentally killed.

DISCUSSION

The results indicate that the hitherto inadequately explained periods of bile pigment overproduction and anemia which have occurred spontaneously in splenectomized bile fistula dogs under observation in this laboratory, are associated with the presence of *Bartonella* bodies in the blood. That bodies were demonstrated but once in one animal (32-74) and irregularly in another dog (31-359), does not exclude the

² The spleen and liver extracts were prepared and furnished to us by Eli Lilly and Company. Spleen extract No. 343 is prepared according to the method of Cohn and Minot (1) which is used in making liver extract No. 343, a fraction potent in pernicious anemia. The preparation of liver extract No. 55, potent in secondary anemia, is described in a previous publication (11) and the spleen extract No. 55 is prepared according to this method.

possible relationship of *Bartonella* to the condition. That animals apparently have individual susceptibility which varies greatly has been pointed out by Kikuth, in descriptions of acute and chronic forms of *Bartonella* infection. The one dog (32-74) in which we failed to get a clear-cut picture of *Bartonella* bodies in the blood was probably highly resistant to the infective agent. As evidence for this is the fact that on two occasions it was inoculated with blood from another animal which showed *Bartonella* bodies, and on neither occasion was there nearly as marked a reaction in the form of anemia as was observed in the other animals.

It is of interest that spontaneous involvement with *Bartonella canis*, while apparently regularly occurring in splenectomized bile fistula dogs, has not occurred in dogs in this laboratory following a splenectomy alone. Other workers have observed such an event in simple splenectomized dogs. Numerous splenectomized dogs have been studied over long periods of time in our anemia colony, and in no instance has there been any evidence pointing to blood destruction by *Bartonella* or by any other agent. The anemia colony, however, consists of dogs raised in the kennels, and at all times completely isolated from contact with any other dogs. This fact may well explain freedom from infection. In our dogs with splenectomies alone, the post-inoculation anemia has not attained the severity of that observed in the splenectomized animals with bile fistulas. The rôle played by the bile fistulas in the determination of the spontaneity and severity of the condition is not understood. Possibly the lack of certain bile constituents plays a part in the lowered resistance of the animals.

Our single test with neosalvarsan agrees with the work of Kikuth, who tested this drug in five dogs.

Our limited experience with the use of spleen extracts suggests that they may have an inhibiting effect on the condition. Further work along this line is in progress.

SUMMARY AND CONCLUSIONS

Splenectomized bile fistula dogs in this laboratory have regularly exhibited spontaneous periods of anemia, which are associated with excessive bile pigment production.

In three out of four dogs, such periods have been shown to be as-

sociated with the presence in the blood of bodies morphologically indistinguishable from descriptions of *Bartonella canis*.

Simple splenectomized dogs have not shown such periods of anemia arising spontaneously. Inoculations of blood containing *Bartonella* bodies into two splenectomized dogs have resulted in intervals of blood destruction associated with the presence in their blood of bodies similar to those in the inoculated blood. Injection of such blood into a splenectomized biliary-renal fistula dog has resulted in a similar picture.

Efforts to cultivate *Bartonella* bodies in artificial culture media have so far been unsuccessful.

Neosalvarsan appears to have a specific sterilizing effect on the condition.

Spleen extract feeding appears to have an inhibiting effect upon the periods of anemia and bile pigment overproduction.

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EXPLANATION OF PLATE 3

FIG. 1. *Bartonella* bodies in blood of a splenectomized bile fistula dog (29-329).

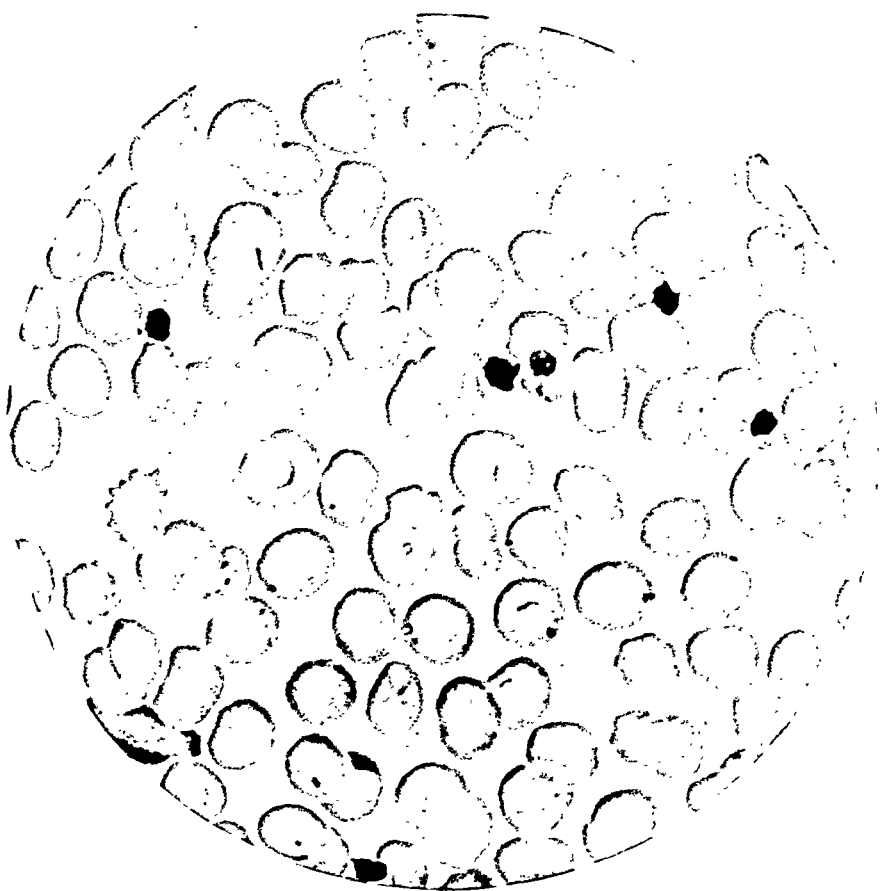


FIG. 1

(Knutti and Hawkins: I. Splenectomized bile fistula dogs)

II. HEMOGLOBIN AND BILE PIGMENT OVERPRODUCTION IN THE SPLENECTOMIZED BILE FISTULA DOG

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In an earlier paper from this laboratory (4), it was pointed out that the splenectomized bile fistula dog showed cycles of bile pigment overproduction and anemia. Because of the very great amounts of bile pigments produced under these conditions we studied this reaction in considerable detail with the hope of a better understanding of body pigment metabolism. Our first paper (4) came to the conclusion that some obscure *intrinsic* factor related to the spleen was responsible for this reaction. The accompanying paper gives strong evidence that an *extrinsic* factor (*Bartonella canis*) is responsible. Splenectomy prepares the way for the *Bartonella* infection.

Granting that *Bartonella* infection explains the blood destruction with resulting surplus bile pigment production, the mechanism of hemoglobin production under these conditions is of peculiar interest to students of anemia. These dogs must produce enormous amounts of new hemoglobin and red cells during an active cycle on a diet which permits of but little new hemoglobin formation in simple anemic dogs. We have suggested that from the released hemoglobin the pyrrol aggregate is split off to form bile pigment while the globin fraction is turned over to form new hemoglobin. This proposal assumes that the body can readily produce a large excess of the pyrrol aggregate (four pyrrol rings) and there is much evidence (1) to support this argument.

To gain further information about blood destruction in bile fistula dogs we carried out a prolonged experiment with acetyl phenylhydrazine (Table 23) which gives similar results and supports the *Bartonella* observations.

Methods

The renal type of bile fistula was used as devised by Kapsinow, Engle, and Harvey (3). This type of fistula has been utilized for several years in this laboratory and the care of these dogs has been described in detail elsewhere (7). We emphasize the fact that such dogs with bile flowing freely into the renal pelvis can be maintained in perfect health and weight equilibrium for years. It is necessary to give 50–75 cc. of bile daily, together with a balanced ration.

The spleen may be removed at the time of the bile fistula operation or subsequently. Dogs are kept in metal metabolism cages at all times and water is given by stomach tube about 3 hours before the 24-hour urinary collection is made. The dog usually empties the bladder between the water ingestion and urine collection which makes for uniformity in urine collection, as obviously residual urine may give irregular values for daily bile pigment elimination. Catheterization would introduce infection and is never employed. Chloroform 5 cc. placed in the collection bottle acts as a preservative.

The dogs are weighed 3 times each week. Red cell hematocrit and blood hemoglobin determinations are made at least once a week and often daily during periods of pigment overproduction and anemia (5). Occasional blood plasma volume determinations are done by the vital red method (2).

Diets are essentially the same in each experiment and consist of canned salmon, Klim (a commercial dried whole milk powder), and a bread prepared in this laboratory. Water 400 cc. is added to the dried bread and the ingredients mixed into a mash. The bread is used in our anemia colony and is an adequate diet capable of maintaining dogs in health indefinitely. It contains wheat flour, bran, potato starch, canned salmon, sugar, cod liver oil, canned tomatoes, yeast, and a salt mixture. Its preparation has been described (10). On this diet the hemoglobin production of anemic healthy dogs has been carefully studied and is well understood. The obvious advantage of this diet in bile fistula dogs for bile pigment study needs no comment. An output of 2–5 gm. of hemoglobin each week over and above the maintenance factor is to be expected on this diet.

Bile Pigment Determination in Urine.—The urine-bile mixture passed in the 24-hour period is measured, and if from previous determinations the amount of bile pigments is known to be high the urine may be diluted with water to a definite volume.

Ten and 20 cc. portions of the diluted or undiluted 24-hour urine samples are made alkaline to litmus with ammonia, then 10 cc. of a calcium chloride solution (10 gm. in 100 cc. of solution) are added. This causes a voluminous precipitate to form, which is thrown down by use of the centrifuge, the supernatant clear fluid is decanted and the precipitate is dissolved in 10 cc. of 95 per cent alcohol to which 2 cc. of concentrated hydrochloric acid are added. The solution is poured into a 50 cc. volumetric flask, concentrated nitric acid containing oxides of nitrogen is added (0.2–0.4 cc.) and then made up to volume with alcohol. The blue-green color develops within a few minutes and the pigments are estimated by means of the colorimeter—Dubosque type.

One of the essential factors of the method is the presence of oxides of nitrogen in the nitric acid. This is of the greatest importance in order to insure proper development of the blue-green color. Pure nitric acid will produce the color reaction but it does so slowly. When the oxides of nitrogen are present in the nitric acid its oxidizing power is greater so that the bile pigments are oxidized to the blue-green phase within a few minutes. Amounts of this acid ranging from 0.2-0.4 cc. prove sufficient for the development of the end-point color and still do not cause the pigment to be oxidized rapidly beyond the blue-green phase. If, upon addition of the above amount of acid, the color development is slow, it may be hastened by immersing the flask in hot tap water with subsequent cooling to the correct volume.

Since the amount of bile pigment in the urine varies for different dogs it necessitates slight variations in the amount of acid needed. By adding the nitric acid 0.1 cc. at a time with a few minutes' interval one can observe the rapidity of color development and easily determine the amount of acid necessary.

The standard for bile pigment is made up 0.3 cc. of a solution of potassium bichromate (1 gm. in 100 cc. of water) plus 40 cc. of a solution of copper sulfate (10 gm. of $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ to 100 of water). This standard solution was described by Rous and McMaster (6) and in this laboratory is standardized against samples of pure bilirubin prepared by Eastman Kodak Company. From this standard solution colorimetric readings enable the investigator readily to estimate milligrams of bile pigment in any solution. The milligram equivalents of bile pigments which this standard solution represents depend on the purity of the sample of bilirubin used in standardization.

A Wratten filter, No. 72, is employed in reading the colors, since urinary pigments themselves give a tint which without the filter make color matching difficult.

In the determination of bile pigments in whole bile, 1 cc. of bile is added to 49 cc. of the acid alcohol reagent in a volumetric flask, mixed, and allowed to stand at ice-box temperature until the blue-green color develops. The time necessary is about 20 hours, but would be shortened if flasks were kept at room temperature. The acid alcohol solution consists of 16 cc. concentrated hydrochloric acid, 2 cc. of nitric acid containing traces of the oxides of nitrogen made up to 1000 cc. with 95 per cent ethyl alcohol.

EXPERIMENTAL OBSERVATIONS

Bartonella Dogs.—Several animals of this type have been studied, and in so far as the blood picture and the pigment values are concerned, the results have been similar. We summarize here studies on one such animal extending over periods of 65 and 134 days. Such periods are characteristic of all animals observed. Chart E shows the bile pigment and hemoglobin variations in a typical animal (31-359). This dog had been operated upon 80 days

previous to the beginning of the chart. The bile pigment and hemoglobin during the postoperative interval had remained within levels corresponding to the first 15 days shown on the chart. The abrupt rise of pigment output and the associated fall of hemoglobin are characteristic, as are the step-like swings of bile pigment to higher

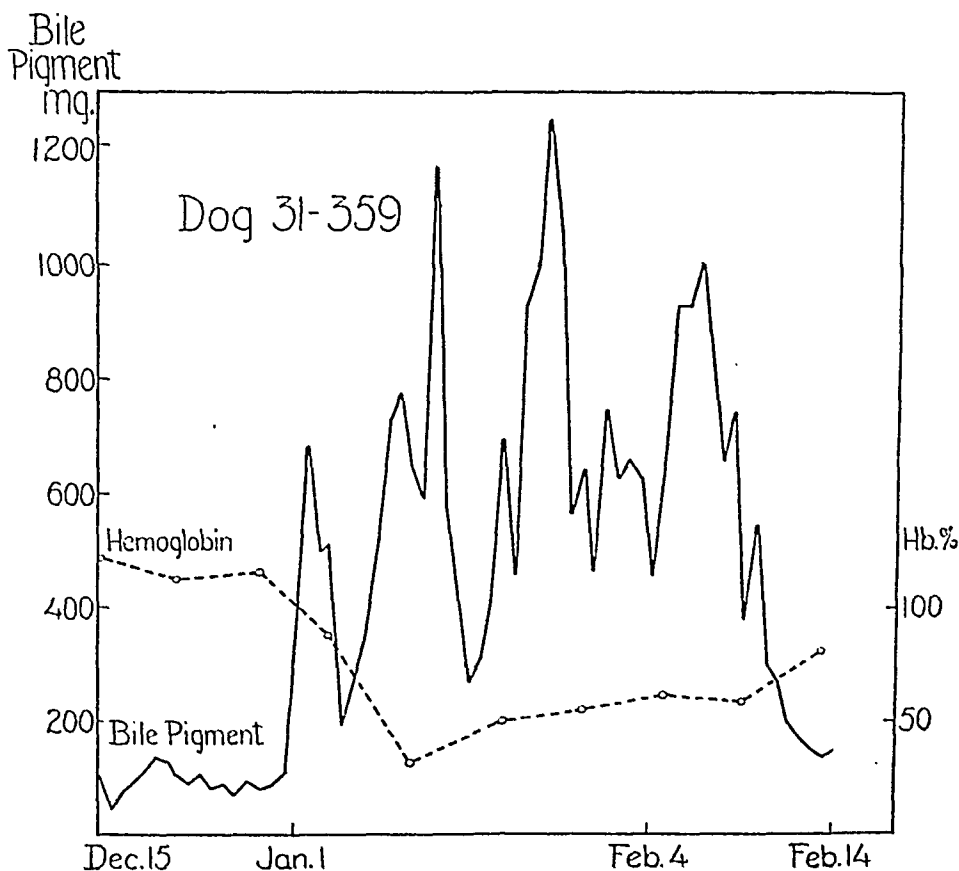


CHART E. Period of bile pigment overproduction and anemia in splenectomized bile fistula dog.

levels. During the period when the hemoglobin was very low, the dog was transfused several times in order to maintain life.

Table 21 is a summary of pigment data of the same dog for 134 days, during which time the animal was showing numerous cycles of blood destruction.

The various periods range, as shown in Column 1, from 6-25 days. They represent phases in which the pigment metabolism was relatively similar. In some of the periods a comparatively small amount of blood destruction was taking place; in others, large amounts of blood were being broken down. Column 2 shows the total amount of bile pigment estimated in the urine during the corresponding experimental period. The excess of pigment above the control level for a similar length of time is shown in Column 3. This control level is obtained by multiplying the number of days of the experiment at hand by the average daily output of bile

TABLE 21
Pigment Surplus in the Splenectomized Bile Fistula
Dog 31-359. Basal bile pigment output 87 mg. per day.

Days of experiment	Total bile pigment output			Blood Hb. level		Pigment lost gm. Hb. equivalent		Pigment gained gm. Hb. equivalent	Pigment surplus gm. Hb. equivalent
	Total	Above control	Ex-pressed as Hb.	Start	End	In urine	From blood	In blood	
	mg.	mg.	gm.	per cent	per cent				gm.
11	1,681	724	18	85	91	18		11	29
6	3,846	3,324	83	91	69	83	39		44
8	2,040	1,344	34	69	81	34		21	55
19	8,666	7,013	175	81	76	175	9	39	166
12	2,085	1,041	26	76	98	26			65
7	1,922	1,313	33	98	96	33	4	7	29
25	4,824	2,659	67	96	100	67		5	74
19	6,622	4,969	124	100	103	124		5	129
8	2,039	1,343	34	103	106	34		5	39
19	5,917	4,264	107	106	111	107		9	116
134	39,642	27,994	701						
Per day.....	295	209	5.2			701	52	97	746
					5.2	0.39	0.72	5.5	

pigment during the control period, which in this case was of 9 weeks' duration. Average basal bile pigment output during this period was 87 mg. daily. The excess amounts of bile pigment expressed as hemoglobin, represented in Column 4, are obtained by dividing the figures in Column 3 by 40, 1 gm. of hemoglobin yielding 40 mg. of bile pigment. Column 7 shows identical figures with Column 4. All subsequent calculations are in terms of grams of hemoglobin. The pigment lost gained in the blood is estimated from the fall or rise in hemoglobin, which is recorded in Columns 5 and 6. The grams of hemoglobin equivalent to the change in hemoglobin percentage are estimated by the formula:

Grams Hemoglobin = $13.8 \times \text{Blood Volume} \times \text{Per Cent Hemoglobin Change}$,

previous to the beginning of the chart. The bile pigment and hemoglobin during the postoperative interval had remained within levels corresponding to the first 15 days shown on the chart. The abrupt rise of pigment output and the associated fall of hemoglobin are characteristic, as are the step-like swings of bile pigment to higher

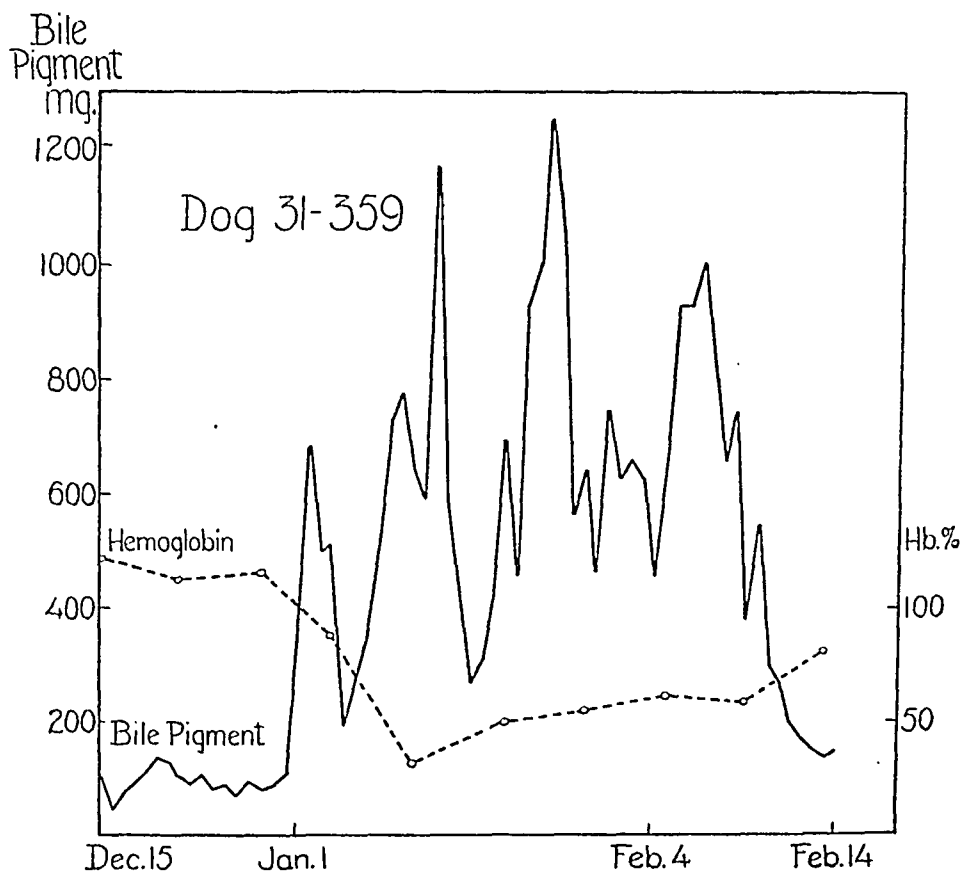


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	Total	Above control	Ex-pressed as Hb.	Start	End	In urine	From blood	In blood	
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12	2,085	1,041	26	76	98	26		39	65
7	1,922	1,313	33	98	96	33	4		29
25	4,824	2,659	67	96	100	67		7	74
19	6,622	4,969	124	100	103	124		5	129
8	2,039	1,343	34	103	106	34		5	39
19	5,917	4,264	107	106	111	107		9	116
134	39,642	27,994	701			701	52	97	746
Per day.....	295	209	5.2			5.2	0.39	0.72	5.5

pigment during the control period, which in this case was of 9 weeks' duration. Average basal bile pigment output during this period was 87 mg. daily. The excess amounts of bile pigment expressed as hemoglobin, represented in Column 4, are obtained by dividing the figures in Column 3 by 40, 1 gm. of hemoglobin yielding 40 mg. of bile pigment. Column 7 shows identical figures with Column 4. All subsequent calculations are in terms of grams of hemoglobin. The pigment lost or gained in the blood is estimated from the fall or rise in hemoglobin, which is recorded in Columns 5 and 6. The grams of hemoglobin equivalent to the change in hemoglobin percentage are estimated by the formula:

$$\text{Grams Hemoglobin} = 13.8 \times \text{Blood Volume} \times \text{Per Cent Hemoglobin Change},$$

100 per cent hemoglobin representing 13.8 gm. per 100 cc. of whole blood. Such differences are shown in Columns 8 and 9, as the pigment lost or gained in the blood.

The pigment surplus is represented in Column 10. This is estimated by the addition or subtraction, as the case may be, of the pigment change in the blood, to or from the excess of bile pigment in the urine. As is seen, the amounts of bile pigment at various periods bear no direct relation to the change in the hemoglobin level during the same periods. In addition, there is no constancy between the figures representing the pigment surplus and either the bile pigment or the hemoglobin values.

In summarizing this table, it is seen that the total time of the experiment was 134 days, during which time the dog eliminated 39,642 mg. of bile pigment. This amount is 27,994 mg. above the normal control level for the animal, and represents an equivalent of 701 gm. of destroyed hemoglobin. During this interval, 97 gm. of hemoglobin were built up, and 52 gm. of hemoglobin were destroyed, as measured in the blood stream. Thus the dog had, at the end of the period, 45 gm. more circulating hemoglobin than at the start of the experiment. This, plus the hemoglobin equivalent for the bile pigment which was eliminated, gives a total pigment excess of 746 gm. This represents a daily average formation of 5.5 gm. of hemoglobin.

Phenylhydrazine Experiments.—Dog 31-267 with a gall bladder-renal fistula was used in these experiments. It was given acetyl phenylhydrazine hydrochloride subcutaneously, in an attempt to determine whether the destruction of blood by this drug would produce pigment changes in any way similar to those of the *Bartonella* animals. This animal was kept under experimental conditions similar to the other dogs.

Table 22 represents a fore period in the course of the experiment, which is followed by an interval in which three doses of 150 mg. each of the drug were given. In this fore period 123 mg. of bile pigment were eliminated daily on an average as compared with the basal control level of 66 mg. This higher pigment level is due to the fact that this period followed a previous injection of phenylhydrazine and the control level had not been attained before more drug was injected. As is shown, the daily bile pigment elimination increases, while the blood hemoglobin percentage falls immediately following the injections. The excess bile pigment above the fore level averages 155

mg. a day which is equivalent to 4 gm. of hemoglobin. During the experimental period there was a drop in hemoglobin from 121 per cent to 80 per cent, which is equivalent to a loss of 44 gm. of circulating

TABLE 22

Acetyl Phenylhydrazine Blood Destruction and Bile Pigment Output

Dog 31-267.

Date	Bile pigment output per 24 hrs.	Hb. level	Urine-bile collected 24 hrs.	Weight	Amount hydrazine injected
	mg.	per cent	cc.	kg.	mg.
1932					
Oct. 16	125		510		
17	138		430	13	
18	105		660		
19	127		495	13.2	
20	127	121	490		
21	158		640	13.2	
22	112		720		
23	96		600		140
24	272		330	13.3	
25	191		720		
26	160		400	13.2	
27	229	104	510		
28	167		890	13.2	150
29	259		450		
30	359		500		
31	309		610	13.1	
Nov. 1	261		540		
2	283	87	500	13.1	
3	191		590		
4	392		450	13.1	150
5	304		570		
6	370		490		
7	467		450	13.2	
8	263		750		
9	256	80	470	13.2	

Diet = 250 gm. salmon bread—food consumption 100 per cent.

75 gm. salmon.

30 gm. Klim.

hemoglobin. (The blood volume of the animal was 952 cc.) 44 gm. of destroyed hemoglobin, theoretically, would cause 1760 mg. of bile pigment above the fore level to be eliminated. The actual recovery

was 2790 mg. or 1030 mg. in excess of the expected amount. Such excess bile pigment elimination is quite similar to that found in the *Bartonella* dogs.

Table 23 is constructed similarly to Table 21, and illustrates the results of continued injections of acetyl phenylhydrazine over comparatively long periods of time. The average dose of the drug was 100 mg. injected subcutaneously every day or two. In the first part

TABLE 23

Pigment Surplus with Hydrazine Blood Destruction in Bile Fistula

Dog 31-267. Basal bile pigment output 66 mg. per day.

Days of experiment	Total bile pigment output			Blood Hb. level		Pigment lost gm. Hb. equivalent		Pigment gained gm. Hb. equivalent	Pigment surplus gm. Hb. equivalent	Hydrazine amount injected
	Total	Above control	Expressed as Hb.	Start	End	In urine	From blood	In blood		
	mg.	mg.	gm.	per cent	per cent				gm.	
36	5,464	3,088	77	128	94	77	44		33	0.24
21	1,383	—3		94	129			45	45	0
23	5,985	4,467	110	104	85	110	39		72	0.60
7	1,400	938	23	85	80	23	7		16	0.20
8	2,109	1,581	40	80	94	40		19	59	0
21	6,817	5,431	135	94	53	135	54		81	0.78
17	1,757	635	16	53	86	16		43	59	0
31	7,520	5,474	137	82	46	137	47		90	1.5
21	5,676	4,290	107	46	57	107		14	121	1.0
35	11,784	9,474	237	57	59	237			237	2.8
21	2,723	1,337	33	59	106	33		62	95	0
241	52,618	36,712	915			915	191	183	908	
Per day.....	218	152	3.8			3.8	0.79	0.76	3.7	

of the experiment a few larger doses of 200–300 mg. were injected but they caused too rapid hemolysis of blood and the dog became upset and consumed only portions of its food.

Comparison of the two tables shows that the pigmentary changes are similar. Again there is no direct relation between the amounts of bile pigment and changes in the hemoglobin level. During the experimental period of 241 days the dog eliminated 36,712 mg. of bile

pigment in excess which is equivalent to 915 gm. of hemoglobin. During this interval 191 gm. of hemoglobin were destroyed and 183 gm. of new hemoglobin were formed as measured in the blood stream. The total excess of pigment formed amounts to 908 gm. expressed as hemoglobin equivalent or a daily average formation of 3.7 gm. of hemoglobin.

In those periods when no drug was injected the bile pigment elimination decreased and there was a rapid increase in the amount of circulating hemoglobin showing how readily the dog could rebuild hemoglobin from the conserved products of destroyed red cells. Undoubtedly new hemoglobin was being formed during the other periods but this fact cannot be brought out from simply measuring the amount of circulating hemoglobin.

DISCUSSION

Hemoglobin production holds the spotlight in this paper and interesting possibilities suggest themselves to explain the observed facts. During active cycles of red cell destruction (*Bartonella*) these bile fistula splenectomized dogs will produce over 5 gm. of new hemoglobin per day or a total of 140 gm. per 2 weeks. In simple anemia dogs this large hemoglobin output (9) can be attained only by a very favorable diet intake (liver plus iron). On the standard basal ration (salmon bread) in simple anemia dogs we expect 5-10 gm. hemoglobin output per 2 weeks. The splenectomized bile fistula dogs are fed this basal ration.

At present the most satisfactory explanation is that the hemoglobin released from destroyed cells is promptly split with escape of the pyrrol aggregate (four pyrrol rings) as bile pigment and the globin fraction is recaptured or turned over to form promptly the new hemoglobin for the red cells in the marrow. The iron presumably goes along alone or with the globin to the make-up of this new hemoglobin.

If one is not wholly satisfied with the preceding explanation it may be argued that not all of the bile pigment comes from hemoglobin but that some of the pyrrol aggregate formed within the body is turned out as excess bile pigment. In any case it must be admitted that the body can supply a large surplus of pyrrol aggregate whether it is all built up into new hemoglobin or some of it is shunted directly into

bile pigment. There are some experimental data which support this last proposal.

Destruction of blood by phenylhydrazine *in vivo* gives a picture much like *Bartonella* blood destruction. It is of similar character but the new hemoglobin output is distinctly less in the hydrazine experiment cited above (Table 23). However we have reported (4) *Bartonella* experiments of only moderate severity which show an identical picture. More severe blood destruction could be induced by hydrazine but the dogs would be sick and refuse food which introduces a confusing factor. The hydrazine experiments support the argument that all or the great bulk of the bile pigment in these experiments comes from hemoglobin.

It is obvious that under the conditions of these experiments, the amount of hemoglobin turnover in the body at any one time cannot be estimated by studies of the blood alone. The ordinary methods for blood hemoglobin determination show the total increase or decrease of this substance in a given time. In the absence of more than normal rates of blood destruction *in vivo*, or in removal of blood from the circulation, such methods offer a satisfactory basis for estimating hemoglobin regeneration. In the presence of excessive blood destruction, however, no accurate idea of the actual amount of pigment turnover can be secured by these methods alone. Under such conditions, hemoglobin may be built up so rapidly, that although comparatively large amounts are destroyed, the blood hemoglobin level in a given interval may show only a slight drop or even an actual rise. In such cases, the estimation of the amount of bile pigment eliminated is of great value.

Other observed facts which are related to this debate should be mentioned. In this laboratory it has been found that in standard anemic dogs hemoglobin introduced intravenously will be quantitatively returned as new hemoglobin in new red cells. This reaction follows whether we use dog or sheep or goose hemoglobin in anemic dogs (8). In the normal healthy bile fistula dog hemoglobin introduced intravenously results usually in quantitative appearance (7) of bile pigment in the proportion of 1 gm. hemoglobin = 40 mg. bile pigment. But in the *anemic* bile fistula dog (1) hemoglobin given intravenously results in an apparent paradox. There is a quantitative recovery of the hemoglobin which appears as new hemoglobin

in the new red cells just as in the standard anemic (non-fistula) dog. There is also almost a quantitative escape of bile pigment just as in the non-anemic bile fistula. There must be a surplus production of the pyrrol aggregate whether this is used in the manufacture of new hemoglobin or of bile pigment or both. This experiment therefore supports the *Bartonella* and hydrazine experiments tabulated above.

The *pyrrol aggregate* (4 pyrrol rings) is the essential nucleus of the pigment radicle (hemin) of hemoglobin as well as of bile pigment and related body pigments. The body can produce considerable excess amounts of this pyrrol aggregate on demand. From what source is it derived? From reserve stores? In long continued anemia it would seem that all pigment stores would have been exhausted when anemia of severe grade ($\frac{1}{3}$ normal hemoglobin) is continued for 3 to 5 to 7 years. From diet? But only in certain peculiar conditions does this surplus appear as the need arises and the diet is strictly uniform at all times. From body synthesis? We favor this probability as the reaction occurs so promptly with the formation of such large amounts when the need is present. The required amino acids presumably would come from the diet but we cannot believe that this intact pigment nucleus can be concealed as such in the diet under consideration.

Globin makes up approximately 95 per cent of the hemoglobin molecule and we believe its importance is in proportion to this figure. The great bulk of the studies dealing with hemoglobin consider mainly the pigment radicle. The observations above indicate that the pigment radicle can be produced readily in abundance. At times iron may be a determining factor. Probably the globin fraction determines the capacity of the body to produce new hemoglobin under many conditions. How and where is globin produced in the body and what materials go into the synthesis of this large molecule? The source and internal metabolism of globin is shrouded in mystery. Any observed facts related to globin within or without the body will be significant and in time should lead to a better understanding of hemoglobin metabolism.

SUMMARY

Blood destruction associated with *Bartonella* or with a drug (hydrazine) in bile fistula dogs yields a large pigment excess. These dogs form large amounts of new hemoglobin and bile pigment on a diet

which permits of but little new hemoglobin production in standard anemic dogs.

When hemoglobin formation and hemoglobin destruction are occurring rapidly and simultaneously, estimations of the percentage of circulating hemoglobin alone, though showing the eventual *total* increase or decrease of this substance, do not permit one to determine the actual amounts formed or destroyed.

It is suggested that the body can produce readily a large amount of the pyrrol aggregate (four pyrrol rings) which may go to form new hemoglobin. At the same time the globin is probably saved from destroyed red cells and turned over into new hemoglobin for new red cells.

It is certain that globin may be a determining factor under certain circumstances in the construction of new hemoglobin for new red cells. Our knowledge about the construction and internal metabolism of globin is extraordinarily limited.

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THE ASSOCIATION OF BARTONELLA BODIES WITH INDUCED ANEMIA IN THE DOG

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PLATE 4

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The great value of the dog in experiments involving the effect of various procedures on hematopoiesis makes important any pathological condition of that animal which has a bearing on blood formation. In the course of experiments designed to produce anemia in dogs by the feeding of diets which are productive of characteristic mucous membrane changes, the spleens of certain of the experimental animals were extirpated. A number of the animals subjected to this procedure developed an anemia of severe grade, associated with the presence of small coccobacilliform bodies in or on the erythrocytes and free in the plasma. The general appearance of these organisms was strikingly, though not wholly, similar to the appearance of the *Bartonella* bodies which are associated with anemia in the human being and in certain lower animals, notably the rat. The severity of the anemia, its characteristic nature, and the resemblance of the bodies to the *Bartonella* bodies, prompted a more detailed study of the phenomenon.

The etiologic rôle of the minute microorganisms associated with Peruvian Oroya fever of human beings was first suggested by Barton (1) in 1909. Strong (2) and his coworkers considered them to be of protozoan nature and suggested the term "*Bartonella*." It remained for Noguchi (3), however, to grow these organisms in pure culture from a fatal case of Oroya fever, and to produce with the cultures disease manifestations in animals. Noguchi (3) also isolated similar organisms from the blood of a splenectomized rat. Lwoff and Vauzel (4) were able to infect mice with intact spleens with *Bartonella*. Lauda (5) studied the anemia of splenectomized rats in which similar inclusion bodies were present in the erythrocytes. Ford and Eliot (6) were able to carry on the condition in successive animal passages. Studies of *Bartonella* bodies in animals have been greatly

which permits of but little new hemoglobin production in standard anemic dogs.

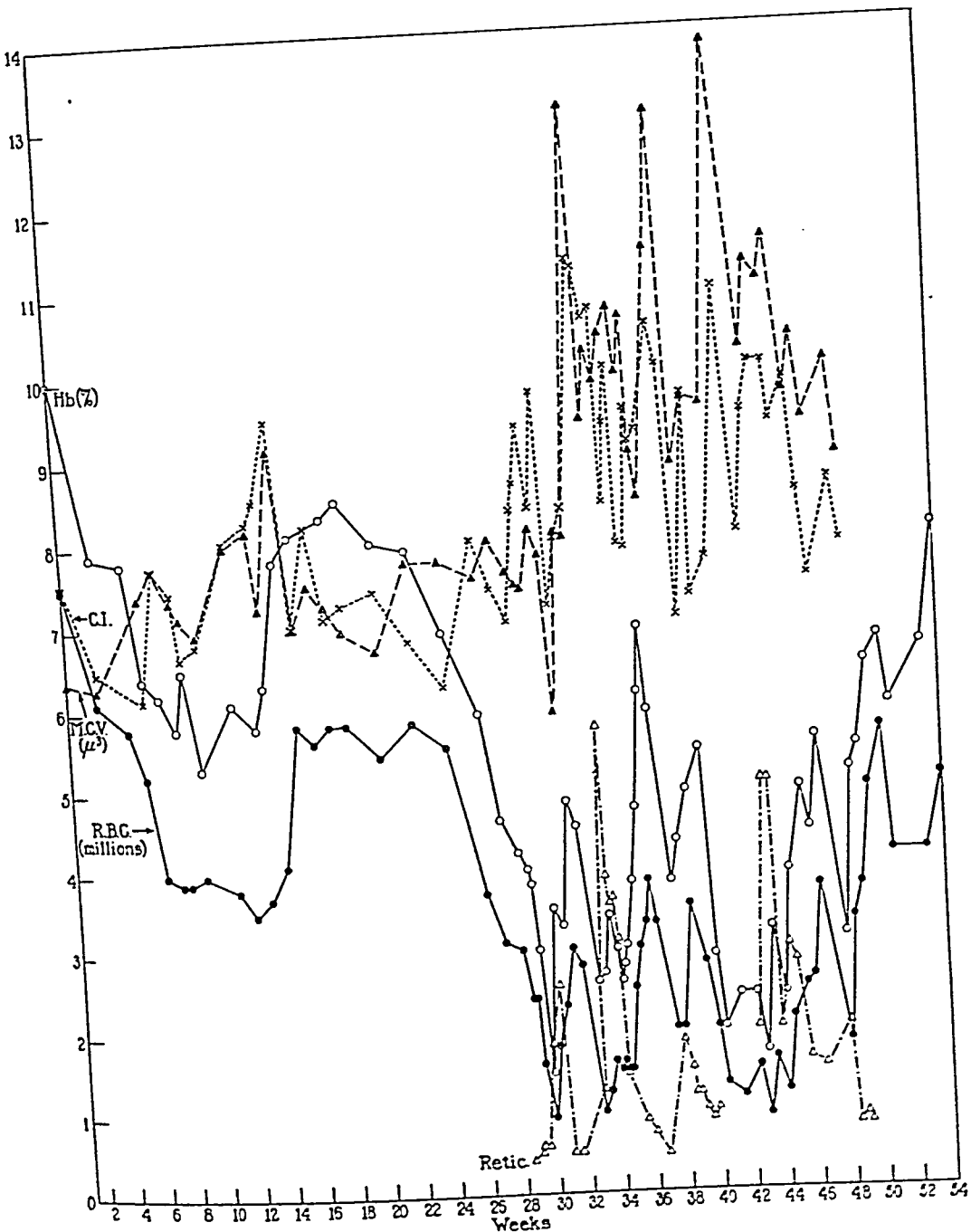
When hemoglobin formation and hemoglobin destruction are occurring rapidly and simultaneously, estimations of the percentage of circulating hemoglobin alone, though showing the eventual *total* increase or decrease of this substance, do not permit one to determine the actual amounts formed or destroyed.

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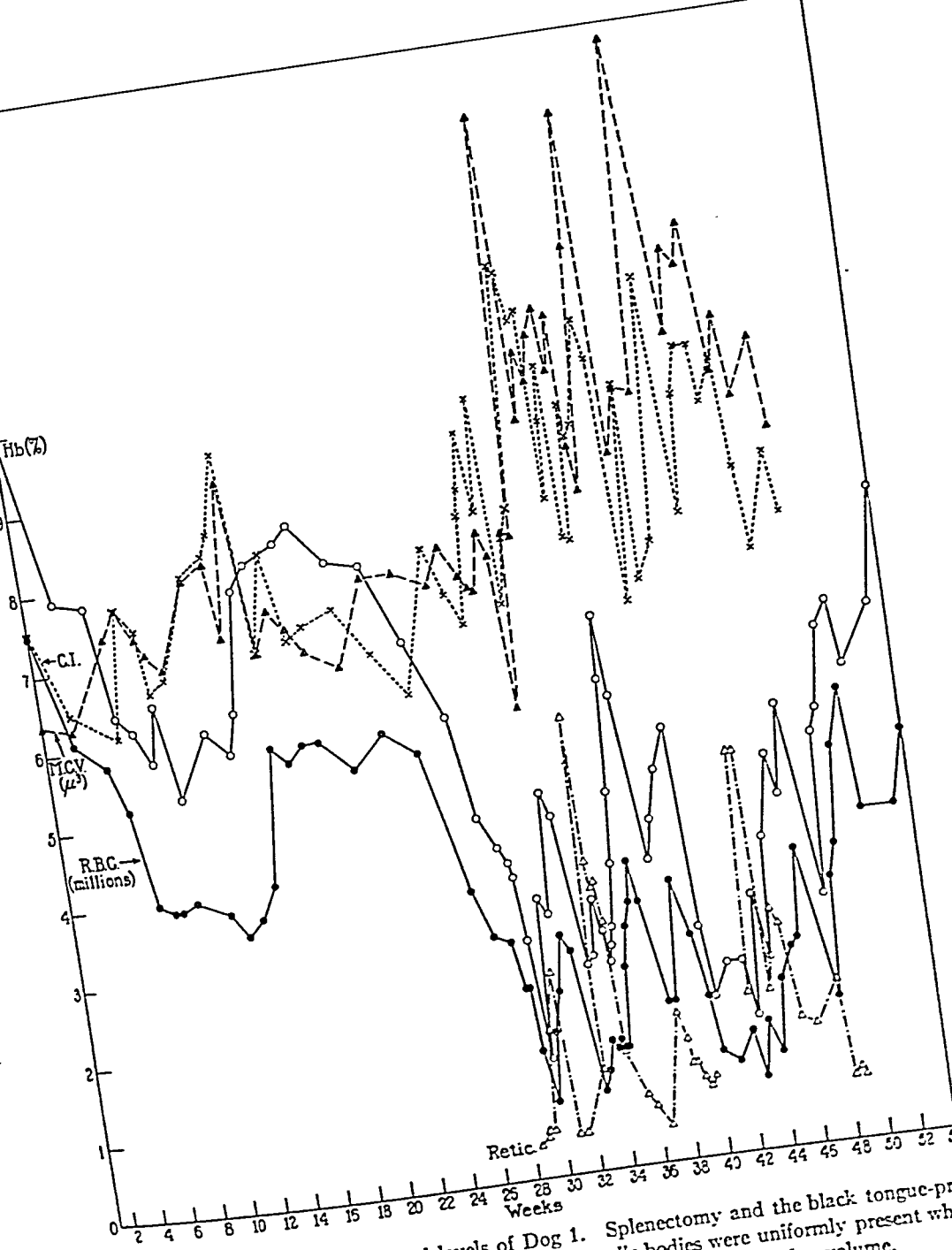
TEXT-FIG. 1. Changes in blood levels of Dog 1. Splenectomy and the black tongue-producing diet were combined in this instance. *Barlone* bodies were uniformly present when the anemia was pronounced. C.I., color index. M.C.V., mean corpuscular volume.

extended by Marmorston-Gottesman and Perla (7). They were able to isolate *Bartonella* bodies in pure culture from the blood of splenectomized rats with *Bartonella muris* anemia, and could cause anemia in other animals by infection with these cultures. They concluded that the adult rat is frequently a carrier of the bodies, although they only appear following splenectomy. These same workers showed that autoplasmic splenic transplants protected against the development of anemia, and that lipoid extracts of the spleen were also effective.

Kikuth (8) gave the first description of *Bartonella* infection in dogs. In a splenectomized dog infected with *Piroplasma canis*, *Bartonella*-like bodies appeared in the blood stream after 6 days. The infection could be transferred to uninfected splenectomized dogs. A chronic type of disease with sharp increases in the number of organisms at regular intervals was described, as well as an acute form followed by death in about 2 weeks. The organisms could not be cultured, but the infection could be cured with salvarsan.

Appearance of Bartonella Bodies in Splenectomized Dogs Fed a Diet Producing Black Tongue

The *Bartonella* bodies of the present observations were first seen in a female terrier weighing 13.7 kilos, which had been splenectomized and fed the modified Goldberger black tongue-producing diet described by Rhoads and Miller (9). During 6 months, a slowly progressive anemia developed. At the end of that period anemia of severe degree appeared suddenly, the number of erythrocytes dropped to below 1,000,000 per c.mm., and the hemoglobin to 15 per cent. Examination of Wright-stained smears of the blood showed a large number of oval to round basophilic bodies present in and on the erythrocytes and free in the plasma. These bodies were morphologically strikingly similar to the *Bartonella* bodies occurring in splenectomized rats. When 200 gm. of lean beef were fed daily, a treatment known to be prophylactic against the effect of the diet in producing black tongue, an increase in reticulocytes occurred followed by a marked rise in the numbers of erythrocytes and in the hemoglobin values. During this remission the *Bartonella* bodies were only rarely demonstrable in the circulating blood. When the meat supplement to the diet was discontinued, however, a marked exacerbation of the anemia followed, during which the *Bartonella* bodies were once more present in great profusion. In Text-fig. 1 are shown graphically the exacerbations of the anemia followed by remissions induced by meat feeding and associated with sharp increases in the number of reticulocytes.



TEXT-FIG. 1. Changes in blood levels of Dog 1. Splenectomy and the black tongue-producing diet were combined in this instance. *Bartonella* bodies were uniformly present when the anemia was pronounced. C.I., color index. M.C.V., mean corpuscular volume.

The disease course of this animal was typical of that of four others splenectomized and fed the diet producing black tongue. A long period of diet feeding was followed by a precipitous drop in blood levels with the appearance of *Bartonella* bodies in the blood stream. Remissions, preceded by reticulocyte crisis, and followed by a marked decrease of the numbers of the *Bartonella* bodies, followed the feeding of lean beef as a supplement to the diet.

Transmission of Bartonella Bodies to Splenectomized Dogs Fed the Black Tongue Diet

The association of the presence of the *Bartonella* bodies with anemia in Experiment 1 suggested an attempt to transmit the conditions to other dogs. Two animals were splenectomized and fed the diet producing black tongue. They were immediately injected intravenously with 3 cc. of citrated anemic blood containing *Bartonella* bodies. The inoculation was repeated three times at 3 day intervals. The changes in the blood levels of one such dog are presented in Table I. A progressive fall in the number of erythrocytes and the percentage of hemoglobin resulted. When the animal was treated with lean beef there was a rise in the number of reticulocytes and an increase of the blood values. *Bartonella*-like bodies were present in large numbers at the height of the anemia and became fewer as improvement took place. They eventually disappeared entirely. The course of the disease in these two dogs was similar in all respects to that observed in Experiment 1, and resembled in many details the anemia associated with *Bartonella* bodies in other animal species. The rapidity of the fall of blood levels precludes the possibility that the anemia resulted from the splenectomy and black tongue diet alone. Furthermore, the results of the next experiment serve as controls on the spontaneous appearance of the *Bartonella* bodies.

Transmission of the Bartonella Bodies to Splenectomized Dogs Fed a Normal Diet

Since in Experiments 1 and 2 variables were present, one an inadequate diet and the other the removal of the spleen, it was deemed desirable to ascertain which factor allowed the *Bartonella* bodies to develop. It was found possible to cause a profound and fatal anemia

irregularly in splenectomized dogs fed a normal diet and injected with blood from Dog 1 containing *Bartonella* bodies. In Table II are presented the hematological findings in one such animal, typical of the four dogs injected.

From these results it is apparent that splenectomy is a major factor in allowing the development of the *Bartonella*-like bodies in the blood stream of the dog once they have been artificially introduced. Krum-bhaar (10), in an extensive series of studies of the hematologic effect of splenectomy in the dog, had failed to note any considerable degree of anemia or the presence of *Bartonella* bodies. Moreover, in our

TABLE I
Dog 2

Date	Erythrocytes per c.mm.	Hemoglobin	Leukocytes per c.mm.	<i>Bartonella</i> bodies
	millions	per cent		
1933	Blood containing <i>Bartonella</i> -like bodies injected			
Mar. 6	5.61	71	6,200	None
	4.28	60	8,250	None
Mar. 13	3.23	56	16,050	None
Mar. 21	3.92	50	13,150	None
Mar. 24	2.23	36	5,300	Few
Apr. 4	2.17	29	10,500	Many
Apr. 7	2.04	33	37,900	Many
Apr. 10	Treatment begun, 200 gm. lean beef daily. Reticulocyte rise to 20 per cent			
	3.30	50	11,000	Few
Apr. 17	4.20	59	11,500	None
Apr. 22	4.30	65	7,800	None
May 3	5.27	61	6,400	None
May 15				

experiments the bodies were never observed in instances in which splenectomy was not combined with the feeding of an inadequate diet. In view of this fact, it was inferred that the diet in our experiments must also have an effect in the production of anemia, an inference which was borne out by the following experiment.

Attempted Transmission of Bartonella Bodies to Non-Splenectomized Dogs Fed the Black Tongue Diet

In Experiment 2 it was shown that by the injection of blood containing *Bartonella*-like bodies it was possible to cause the appearance

of similar bodies, and, also, anemia in splenectomized dogs fed a diet causative of black tongue. Moreover, the previous experiment was evidence that the diet feeding was not necessary to allow the development of the transmitted disease, since splenectomy alone was sufficient

TABLE II

Dog 3

Date	Erythrocytes per c.mm.	Hemoglobin	Leukocytes per c.mm.	<i>Bartonella</i> bodies
1933	millions	per cent		
Mar. 9	6.83	95	6,800	None
Mar. 20	5.18	81	29,200	None
Mar. 27	6.07	81	11,650	None
Apr. 4	3.60	54	13,250	Few
Apr. 10	1.02	13	22,350	Enormous number
Apr. 13	1.17	22	24,900	Enormous number
Apr. 16	0.64	10	45,900	Enormous number
Apr. 21	0.70	11	30,100	Enormous number

TABLE III

Dog 4

Date	Erythrocytes per c.mm.	Hemoglobin	Leukocytes per c.mm.	<i>Bartonella</i> bodies
1933	millions	per cent		
Feb. 22	3 cc. of blood from Dog 1 containing many <i>Bartonella</i> -like bodies injected intravenously			
	493	70	22,900	None
Mar. 6	474	71	10,400	None
Mar. 13	437	65	—	None
Mar. 21	481	61	14,000	None
Mar. 27	417	59	13,850	None
Apr. 4	371	50	20,800	None
Apr. 10	329	44	14,700	None
Apr. 13	292	44	13,400	None
Apr. 17	277	46	13,700	None

to allow the effect to occur. It was desirable to ascertain next whether the diet, without splenectomy, was sufficient to allow the proliferation of the bodies in the blood stream. To answer this question, two dogs which were fed the diet producing black tongue, but which had not

been splenectomized, were injected with blood of Dog 1 containing large numbers of the bodies. The results are shown in Table III. There occurred a progressive decrease in blood levels, described by Rhoads and Miller (9) as an effect of the diet feeding alone, if persisted in for a sufficiently long time to cause chronic stomatitis to appear. At no time, however, were any *Bartonella* bodies to be found in the blood stream, a fact in striking contrast to the results of the two previous experiments. It was concluded, therefore, that although the diet feeding alone would cause anemia, it did not suffice of itself to allow proliferation of the *Bartonella*-like bodies. The course of the changes of the blood of one such animal is presented in Table III.

Attempted Transmission of Bartonella Bodies to Non-Splenectomized Dogs Fed a Normal Diet

Two normal dogs fed a diet used in this laboratory for many years and known to be adequate to maintain dogs in good health for an indefinite period, were injected intravenously with 20 cc. of blood from Dog 1 containing many *Bartonella* bodies. At no time did those bodies appear in the blood of the recipient animal, nor did any decrease of blood levels take place.

Attempted Transmission of Bartonella Bodies to Other Animals

Marmorston-Gottesman reported that she was able to cause anemia and the appearance of *Bartonella muris* bodies in the blood of a variety of animals by the injection of cultures of that organism.

Mice, white rats averaging 30 gm. in weight, young rabbits, puppies, and young guinea pigs were all injected intraperitoneally with dog blood containing the *Bartonella* bodies. In no instance did symptoms of disease result or anemia appear, nor were the bodies found in the blood stream. So far as the evidence goes, it favors the view that the bodies seen in the dog, though morphologically similar to those occurring in the splenectomized rat, differ radically from them in that they cannot infect other animals.

Morphology of the Bodies

In smears of blood stained by Wright's method, the bodies are mostly present in or on the red corpuscles, but are occasionally found

of similar bodies, and, also, anemia in splenectomized dogs fed a diet causative of black tongue. Moreover, the previous experiment was evidence that the diet feeding was not necessary to allow the development of the transmitted disease, since splenectomy alone was sufficient

TABLE II

Dog 3

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Mar. 9	6.83	95	6,800	None
Mar. 20	5.18	81	29,200	None
Mar. 27	6.07	81	11,650	None
Apr. 4	3.60	54	13,250	Few
Apr. 10	1.02	13	22,350	Enormous number
Apr. 13	1.17	22	24,900	Enormous number
Apr. 16	0.64	10	45,900	Enormous number
Apr. 21	0.70	11	30,100	Enormous number

TABLE III

Dog 4

Date	Erythrocytes per c.mm.	Hemoglobin	Leukocytes per c.mm.	<i>Bartonella</i> bodies
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Apr. 17	277	46	13,700	None

to allow the effect to occur. It was desirable to ascertain next whether the diet, without splenectomy, was sufficient to allow the proliferation of the bodies in the blood stream. To answer this question, two dogs which were fed the diet producing black tongue, but which had not

been splenectomized, were injected with blood of Dog 1 containing large numbers of the bodies. The results are shown in Table III. There occurred a progressive decrease in blood levels, described by Rhoads and Miller (9) as an effect of the diet feeding alone, if persisted in for a sufficiently long time to cause chronic stomatitis to appear. At no time, however, were any *Bartonella* bodies to be found in the blood stream, a fact in striking contrast to the results of the two previous experiments. It was concluded, therefore, that although the diet feeding alone would cause anemia, it did not suffice of itself to allow proliferation of the *Bartonella*-like bodies. The course of the changes of the blood of one such animal is presented in Table III.

Attempted Transmission of Bartonella Bodies to Non-Splenectomized Dogs Fed a Normal Diet

Two normal dogs fed a diet used in this laboratory for many years and known to be adequate to maintain dogs in good health for an indefinite period, were injected intravenously with 20 cc. of blood from Dog 1 containing many *Bartonella* bodies. At no time did those bodies appear in the blood of the recipient animal, nor did any decrease of blood levels take place.

Attempted Transmission of Bartonella Bodies to Other Animals

Marmorston-Gottesman reported that she was able to cause anemia and the appearance of *Bartonella muris* bodies in the blood of a variety of animals by the injection of cultures of that organism.

Mice, white rats averaging 30 gm. in weight, young rabbits, puppies, and young guinea pigs were all injected intraperitoneally with dog blood containing the *Bartonella* bodies. In no instance did symptoms of disease result or anemia appear, nor were the bodies found in the blood stream. So far as the evidence goes, it favors the view that the bodies seen in the dog, though morphologically similar to those occurring in the splenectomized rat, differ radically from them in that they cannot infect other animals.

Morphology of the Bodies

In smears of blood stained by Wright's method, the bodies are mostly present in or on the red corpuscles, but are occasionally found

free. They are round to oval in shape and measure from 0.2 to 0.4 micron in width. The long forms may attain a length of 6 microns, but it is impossible to state whether these are single organisms or chains of smaller forms. Bent forms are frequently present. The longer bodies frequently show a regular indentation of the lateral border, giving the appearance of chains of smaller bodies. This is not always the case, however, since perfectly straight sided forms have been observed. The bodies do not stain with gentian violet when stained by Gram's method and when studied by dark-field illumination they are actively motile.

DISCUSSION

The experiments reported suggest the conclusion that both the diet producing black tongue and splenectomy favor the appearance of anemia together with *Bartonella* bodies in the blood of the dog. Neither procedure alone has resulted in this association. If blood containing the bodies is injected into splenectomized animals fed either a good or a deficient diet, anemia develops and the bodies appear in great number. This did not occur in any dog from which the spleen had not been removed. It is conceivable that the black tongue diet reduced the natural defense, which had already been lowered by splenectomy, almost to the threshold at which the *Bartonella* bodies appear.

From the morphologic resemblance between the bodies seen in the blood of the animals reported in this communication, and those reported by Kikuth in the dog and termed *Bartonella canis*, the conclusion is unavoidable that they are the same. This is borne out by the similarity of the associated blood dyscrasia.

The fact that the administration of lean beef, a substance known to be preventive of the symptoms which result from the diet feeding, was followed by increases in the number of reticulocytes and improvement of the blood levels, is of particular interest, since it provides evidence that the diet feeding was in part, at least, causative of the hematological disorder. From the studies of Goldberger and his coworkers, it has been assumed that beef is potent in preventing the pathological manifestations resulting from the diet feeding because of its content of vitamin B₂ (G)—a vitamin which may be defined as that heat-stable

fraction of the vitamin B complex which is required to allow growth in rats. But experiments performed in this laboratory have shown clearly that the diet is wholly adequate for rat growth when fed alone, while, furthermore, it has been impossible to cause black tongue in dogs by feeding diets lacking various parts of the vitamin B complex. It seems probable, then, that the effect of the diet is not due to a lack of the rat growth-promoting vitamin, although positive proof is lacking of the way in which it produces disease manifestations.

Proof of the etiologic relationship of the bodies to the anemia has not been presented. The association of the two is suggestive, but some unrecognized anemia-producing agent may have been introduced with them, while in the transfer experiments conceivably the anemic state may have favored multiplication of the bodies. The similarity of the pathological picture with that following *Bartonella* infection in other animals, however, suggests strongly that the bodies caused the anemia when the ground had been suitably prepared by splenectomy.

SUMMARY AND CONCLUSIONS

On feeding to splenectomized dogs a diet producing black tongue, severe anemia developed associated with the presence of small bodies in or on the erythrocytes. The bodies were morphologically similar to *Bartonella muris* and *Bartonella canis*. The addition of lean beef to the diet, a material prophylactic against its effects, was followed by improvement of the blood levels, the presence of increased numbers of reticulocytes in the circulating blood, and the disappearance of the bodies.

When the blood containing *Bartonella*-like bodies was injected into other splenectomized dogs fed a normal or a black tongue-producing diet, anemia developed in them and the bodies appeared in large numbers. Similar injections into non-splenectomized animals fed in the same way had anemia alone as its result. Injections into normal animals fed a normal diet caused not even anemia.

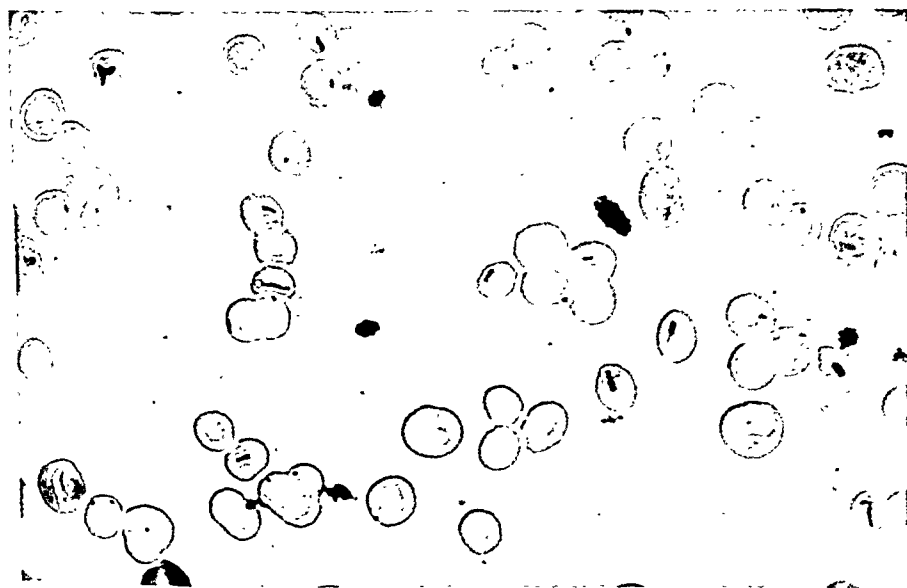
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EXPLANATION OF PLATE 4

FIG. 1. Photomicrograph of a smear of the blood of Dog 1 taken at the height of the anemia. Many *Bartonella* bodies are present. The large dark areas are masses of platelets. Wright's stain. $\times 1500$.



Photographed by Louis Schmidt

FIG. 1

(Rhodospirillum rubrum, B. abortus bodies and infected anemia)

STUDIES ON ANAPHYLAXIS WITH POLLEN

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(Received for publication, November 5, 1934)

Many fundamental questions regarding immunobiological reactions are as yet unanswered. However, certain working principles have been reached through long observation and research, and the terms foreign protein reactions, complement, precipitins, antibodies, anaphylaxis, etc., have acquired definite and specific significance. Attempts have been made to fit these concepts to clinically observed phenomena, but to date the so called pollen sensitizations have never been satisfactorily elucidated on an immunological basis. Many observers have noted the wide variations in the clinical results of therapy. Others have questioned the expectation of any significant effects from the injection of so little real protein in desensitization and have found it difficult to correlate this desensitization and clinical improvement with a skin test persistently positive to the specific atopic agent. The apparent inability to obliterate these hypersensitivities, as well as their frequent multiplicity are problems as yet unexplained.

Efforts at solving these problems have resolved themselves into (a) attempts to sensitize animals and reproduce the syndrome observed in human beings (1); (b) the study of Dale preparations of uterine strips taken from sensitized guinea pigs (2); (c) efforts to sensitize animals by upper air passage or intratracheal insufflation (1); (d) the investigation of serum reagins by Prausnitz-Küstner reaction and by precipitin tests (3); (e) studies of results of therapy in clinical cases of atopic hypersensitiveness (4). These investigations are well known to readers of the literature on the subject. As with all biological experiments the results have been inconstant, or the methods introduced elements into the problem not germane to the investigation at hand.

These and many other considerations led us to inquire into the possible mechanisms involved. What substances in the realm of immunological reagents might reproduce such a series of phenomena? Landsteiner and van der Scheer (5), Avery and Goebel (6, 7), and others, following Landsteiner's original hapten concept, investigated the properties of certain synthesized antigens. They found that the antigen was essentially dual in structure, a larger portion possessing antigenicity, and a smaller, prosthetic portion (hapten), specificity. The union between these two was not close; there was either a loose chemical bond, or a simple colloidochemical linkage. The specific hapten portion, when united with either antigenic nucleus (or haptophore group), was capable of calling forth the specific response, whereas the hapten portion alone, while it could elicit a positive skin reaction (*e.g.* the pneumococcus specific soluble carbohydrate), actually inhibited the union of antigen and antibody *in vitro*.

It is possible that the mechanism involved in atopic hypersensitivity represents just such a hapten-like action, rather than the tacitly assumed, firmer union of true antigen-antibody complex. Certainly atopens as a rule are protein-poor substances; they elicit cutaneous evidence of sensitivity, but in general are ineffective in calling out demonstrable precipitins. However, they give a positive passive transfer phenomenon. Landsteiner has shown that these haptens may be carbohydrates, lipoids, or relatively much smaller proteins. Does some such non-specific antigen produce the allergic state referred to as underlying sensitivity, or antibody unsaturation, or protoplasmic instability (8)? Or shall we be content to accept it as some change (Kahn (9)) which is "rooted in the biologic and physicochemical structure of the chromosomes"?

It was felt that an experiment might be planned which would throw some light on a few of these questions. Various proteins were employed as basic sensitizers, always using a 1:25 extract of burweed marsh-elder (*Iva*) as the superimposed atopen. Experiments were done on several series of guinea pigs: one with veal broth; two with egg white; and one (the present), with horse serum. This last was found to give the most consistent and clear-cut results. We report in detail the final series which constitutes a representative study.

Materials and Method

The guinea pigs were all males, weighing between 300 and 500 gm. Injections, with Luer syringes and $\frac{3}{4}$ inch, 26 gauge needles, were made intracutaneously with some possible subcutaneous leakage. All injections were 0.4 cc. in quantity.

Normal horse serum was obtained from Eli Lilly Co.; the extract of burweed-marsh-elder (*Iva xanthifolia*) was made in our laboratory as follows:

KH ₂ PO ₄	0.54 gm.
Na ₂ HPO ₄	2.26 gm.
NaCl.....	9.00 gm.
Phenol.....	2.5 gm.
Glucose.....	25.0 gm.
Water, sufficient to make.....	500.0 cc.

To each 100 cc. of solvent was added 4 gm. of clean pollen (1931). The mixture was shaken for 24 hours, centrifuged, and the supernatant fluid filtered through standard, fine filter paper. This was next filtered through a Seitz filter and tested for bacterial growth; found sterile, it was kept on ice when not in use.

The mixture of equal volumes of horse serum and pollen extract was allowed to stand at room temperature for 24 hours; at various times during the 3 weeks previous to use it was taken from the ice box for repeated room temperature exposures. The mixture produced no precipitate.

Throughout the experiment separate syringes were used for each material. These were recorded by their serial numbers etched on the barrel and plunger, and their identities constantly maintained. After use they were cleaned separately.

Quantitative analysis (Kjeldahl) of the horse serum showed it to contain 7.35 gm. per cent protein. Average determinations of protein content of the *Iva* extract yielded about 50 mg. per cent. In the latter there were wide variations, due either to the batch of extract, or the method of analysis.

3 weeks after the final sensitizing injections blood samples were drawn by venipuncture for precipitin titration with *Iva* and horse serum, and shock experiments done with both of these substances. For this the jugular vein was exposed, blood first taken by syringe, and 0.4 cc. of the shocking material slowly injected. Each animal was observed for a minimum of 1 hour following this procedure, and the reactions carefully recorded.

EXPERIMENTAL

Six guinea pigs were sensitized by three injections (0.4 cc. each) of whole horse serum at intervals of 4 days. After 3 weeks these (Group B.) and six normal guinea pigs (Group B) were given three injections (0.4 cc. each) of pollen

extract (*Iva xanthifolia*), at 4 day intervals. At the same time twelve normal guinea pigs were injected with three inoculations (0.4 cc.) of equal parts of horse serum and pollen extract (Group A), and a fourth group (Group C) of six animals were sensitized by injections of horse serum alone (0.4 cc.). 3 weeks after the final inoculation the precipitins for pollen extract and for horse serum were investigated, and the anaphylactic response to these substances studied. These are set forth in Table I.

As shown in the table, marked differences were obtained between the several groups of animals, most notably in their response to the test of anaphylaxis. All the guinea pigs of Groups A and B₀ which had received both horse serum and pollen, reacted to intravenous pollen extract with moderate to severe anaphylaxis. One such animal, No. 1-3, was shocked fatally (4+), and only one, No. 1-2, showed a negative response, having received only half the regular shock dose (or 0.2 cc.).

In Group B (animals prepared with pollen extract alone), anaphylaxis to pollen extract could not be demonstrated, even though in one instance the intravenous dose was doubled. It might be pointed out that the absolute amount of pollen extract given in the preliminary injections in this group was actually twice that received by animals of Group A.

Anaphylaxis to horse serum was almost uniformly severe in Groups A, B₀, and C. One animal, No. 9-1, showed only a mild reaction. Group B pigs were quite negative to intravenous horse serum on several trials, and pollen extract educed no response in the animals sensitive to horse serum alone (Group C).

The skin tests in all groups of this series were inconclusive. However, in the three previous series, at a similar date the skin reaction to pollen extract intracutaneously was by objective measurement definitely greater in the Group A animals than in those of Group B. There were no Group B₀ pigs in the earlier experiments.

Precipitin tests in all four series were carried out in the manner described by Hektoen. The results were not sufficiently conclusive to permit any inferences. They are reported, however, as a matter of possible investigative interest.

DISCUSSION

By means of the above and similar experiments we have shown that in the presence of an underlying sensitization, a substance not in

TABLE I

Series IV

Group A. Pollen-horse serum, equal parts.
 Group B. Pollen extract alone.
 Group C. Horse serum alone.
 Group B₀ received same injections as Group B, but 4 weeks after injections of horse serum, 0.4 cc. on July 16, 20, 24.

0.4 cc. injected Aug. 21, 24, and 29.

Group	Animal No.	Precipitin test for		Anaphylaxis to		Remarks
		Pollen	Horse serum	Pollen	Horse serum	
A	1	Neg.				
	2	Pos. (wk.)	Pos. (wk.)			
	3	Pos.	Pos.	++	+++	
	4	Pos. (++)	Pos.	+++		
	5		Pos. (+)	+		
	8			++		
	1-2	Pos.	Pos. (?)	+		
	1-3			Neg.		
	1-4	Pos. (+)	Pos. (+)	++++		Received 1/2 shock dose
	1-5	Pos. (+)	Pos. (++)	++++		
B	1-6	Pos.	Pos.	++++		Pos. Schwartzman phenomenon
	1-0			++++		
	1-1	Pos. (wk.)	Pos. (wk.)	++++		
	1-8	Neg.	Neg.			
	2-4			Neg.		
	2-5			Neg.		
B ₀	2-2			++		
	8-9					
	9-0	Neg.		++		
	9-1	Neg.	Neg.	+++		
	9-2			++		
C	9-3		Neg.	+		
	1-9			++		
	7-7			++		
	9-6	Neg.				
	9-7		Pos.	+++		
	9-8	Pos. (?)	Neg.	++++		
	1-00	Neg.	Pos.	++++		

++++ = lethal, with jactitation, convulsions, etc.
 +++ = dyspnea, convulsions, prostrations with survival.
 ++ = dyspnea, cyanosis, wheezing, cough, loss of posture.
 + = cough, sneeze, scratching of nose, cyanosis, slight dyspnea, micturition, defecation, nervousness.
 precipitin test—wk. = turbidity only slightly greater than control.

itself capable of sensitizing can induce the hypersensitive state, and, when properly administered, produce anaphylactic shock. Hektoen (10) and Parker (11) have both done similar experiments but reported no tests on the anaphylactic response.

Rothschild, Friedenwald, and Bernstein (12), working on desensitization of tuberculin-sensitive guinea pigs (sensitized by inoculation with avirulent strain of Koch's bacillus), found that Koch's old tuberculin (O.T.) in suitable doses could produce complete desensitization by all the criteria they were able to apply. They found also that the broth control produced almost as complete a desensitization, whereas a tuberculoprotein (Seibert) of equal skin test potency produced very little diminution in sensitivity. The difference apparently lay in the beef protein content of the O.T. This finding suggests the important rôle that non-specific immunological reactions may play in the evolution and subsequent course of the allergic state. In this connection may be mentioned the frequent improvement noted in patients with allergic disease during acute infections, and following vaccines and non-specific protein therapy.

How closely this experimentally induced condition approximates the actual clinicoimmunologic mechanism can only be surmised. Further work along these lines is being undertaken with a view to clinical application.

SUMMARY AND CONCLUSIONS

1. Guinea pigs injected intracutaneously and subcutaneously with extract of the pollen of burweed marsh-elder in relatively small amounts did not show anaphylactic response to intravenous shock doses of this material 3 weeks later.

2. If, however, the animals were sensitized with horse serum either before, or along with the same pollen injections, they could then be shocked after an interval of 3 weeks with pollen extract alone.

3. The possible rôle of this underlying sensitivity is discussed.

The author wishes to express his appreciation of the helpful criticism given by Dr. O. H. Robertson.

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SALT AND WATER LOSSES IN DIURETIN DIURESIS AND THEIR RELATION TO SERUM NON-PROTEIN NITRO- GEN AND ELECTROLYTE CONCENTRATIONS

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Gruenewald (1) in 1909 in experiments on rabbits showed that the repeated administration of diuretin caused an excessive chloride loss in the urine and that after the fourth or fifth administration of diuretin the animal died in a paralytic comatose state with strikingly low blood chloride values. Since simultaneous saline administration with each dose of diuretin saved the lives of the rabbits, he felt he had in this way excluded a possible toxic effect of the drug. In repeating this experiment Bilbao and Grabar (2) in Blum's clinic observed that a high blood urea concentration accompanied the low blood chloride and that rabbits supplied with salt solution did not develop azotemia. They concluded that the appearance of azotemia was connected with the fall in blood chloride. Blum (3) in considering the etiology of azotemias in patients without anatomical kidney lesions, assumed that the elevated blood urea was a compensatory mechanism preventing an osmotic deficit in the blood in the presence of low blood chloride. This idea of an etiological relation between low blood chloride and high blood urea concentrations has profoundly influenced the continental literature of the past 5 years. Many clinical and experimental non-nephritic uremias following diarrhea, vomiting, or salt losses through other channels have been reported as "azotemias because of the lack of salts," Blum (3), Ambard (5), Rathery (6), "uremias of chloride want" Strauss (7), Glass (8), and "hypochloremic comas" Porges (9). The fact that administration of saline solution to such patients reduces the azotemia and restores the urea concentrating

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capacity of the kidneys, which Chabanier (10), Glass (8), Meyer (11), and others had observed to decrease with the low serum chloride, is used to support the belief in the etiological relationship between chloride level and nitrogen retention.

Against this view may be cited (see Table III) various conditions produced experimentally or by disease in which an apparently non-nephritic azotemia exists in the presence of a normal or even a much elevated plasma chloride and, conversely, in which large reductions of plasma chloride unaccompanied by nitrogen retention are found. The Blum theory rests almost entirely on measurements of chloride and non-protein nitrogen levels in the plasma. Further and more comprehensive study of water and electrolyte metabolism in relation to impaired nitrogen excretion is therefore desirable. We have accordingly undertaken to repeat Gruenwald's (1) experiment with the purpose of simultaneously following the metabolism of sodium, potassium, chloride, nitrogen, and water and the changes in the serum concentrations of these substances.

Methods

Rabbits weighing between 1730 and 2230 gm. were used. In the earlier experiments the animals were fed according to Gruenwald's (1) original procedure with maize, ground and washed thoroughly with distilled water to reduce the salt content. Since little of this ration was taken, the animals in later experiments were fasted. Every day or, in some instances, every other day, 0.5 gm. of diuretin per kilo of body weight, dissolved in distilled water, was administered with a stomach tube. The animals had free access to water, the amount taken being accurately measured. All blood samples were drawn from the ear veins 24 hours after the preceding administration of water, diuretin, or salt. Care was taken to avoid excitement or movement of the animals as exercise of only short duration has been shown to depress the bicarbonate and cause considerable rise in the fixed base content of the blood serum (Csapó and Kerpel-Fronius (12)).

The urine was collected in 24 hour specimens under toluene, the bladder being emptied by careful manual pressure at the end of each collection period. The analytical methods were as follows: sodium in blood serum and urine according to Butler and Tuthill (13); potassium according to Fiske (14); chloride by Fiske and Lin (15); bicarbonate, manometrically (16); N.P.N. colorimetrically with Nesslerization; and total protein and nitrogen by the Kjeldahl method.

EXPERIMENTAL RESULTS

Fig. 1 presents for each of five fasting animals over 9 day periods the total, not daily, amount of sodium, potassium, and chloride excreted in the urine above the

amount of each administered and also the weight loss, all values being divided by the body weight to reduce them to a standard 1 kilo animal. The values of the scale on the left of the figure represent milli-equivalents, the values of the scale on the right represent weight loss in grams. The losses of each substance in the different experiments are grouped together in the figure. The first two columns of each group are from Experiments 1 and 2 on fasting controls. Column 3 in each group represents the loss in Experiment 3 from a fasting animal receiving four doses of diuretin. Column 4 represents the loss in Experiment 4 from a fasting animal receiving four doses of diuretin plus forced water amounting to 150 to 200 gm. daily in one single dose. Column 5 in each group represents the loss in Experiment 5 from a fasting animal receiving four doses of diuretin plus 70 cc. of saline intraperitoneally on the days of diuretin administration. The weight loss attributable to the nitrogen excretion in the urine was estimated by assuming that each gram of nitrogen represents 29.5 gm. of tissue. The weight loss due to this destruction of protoplasm is represented in Fig. 1 by the darkened portion of each weight column. The extent of the columns above the darkened portions measures the sum of the other factors of the total weight loss. These are: (1) loss of glycogen; (2) loss of body fat; (3) the portion, probably small, of destroyed protoplasm represented by nitrogen excreted in the stools, which was not measured; and (4) water withdrawn from the body above that accompanying the destruction of protoplasm, *i.e.* the fraction of the total weight loss which can be credited to dehydration.¹ The first three factors may be assumed to have approximately the same total value in all of the experiments, since all of the animals were fasted and since no difference in the quantity or consistency of the stools was observed. The extent to which the undarkened portions of the columns in Experiments 3, 4, and 5 are above the average value found in the two control experiments will, therefore, provide a rough measurement of the dehydration produced in these experiments beyond that due to fasting.

From Fig. 1 it can be seen that there is an excessive sodium and chloride-loss in Experiments 3 and 4. The administration of saline in Experiment 5 markedly reduced the loss of these two ions, the actual sodium loss being no greater than in the control animals. The potassium loss was increased above the control level in all three diuretin experiments. The administration of saline did not protect the rabbit of Experiment 5 from a potassium loss double that of the controls. The weight loss attributable to tissue destruction is only slightly higher in the diuretin experiments than in the controls. The weight

¹ The term dehydration as used in this paper means, then, loss of water above that attributable to protoplasm destruction as measured by nitrogen excretion. Thus it includes loss of intracellular fluid from tissue without cell destruction, loss of extracellular fluid, and insensible fluid loss.

loss attributable to dehydration is markedly greater in the diuretin experiments, being the greatest in Experiment 3 where the sum of

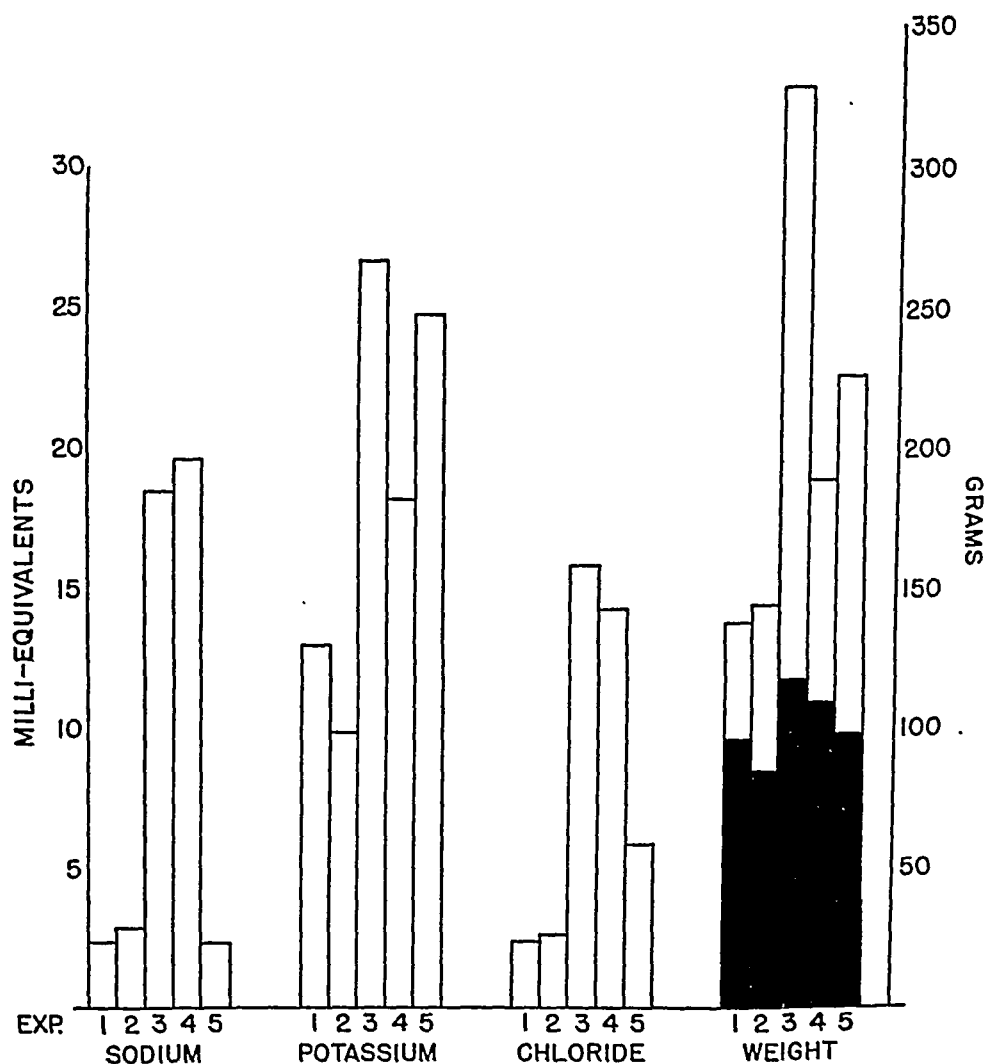


FIG. 1. Urinary excretions of sodium, potassium, and chloride above amount of each ingested and weight losses of fasting animals over a 9 day period. Darkened portion of each weight column represents weight losses attributable to protoplasm destruction as calculated from urinary nitrogen excretion. Experiments 1 and 2, fasting controls. Experiment 3, fasting rabbit receiving diuretin. Experiment 4, fasting rabbit receiving diuretin plus forced water. Experiment 5, fasting rabbit receiving diuretin plus saline.

sodium plus potassium loss is largest. The markedly lower dehydration weight loss in Experiment 4 as compared to Experiment 3 is

associated with both a slightly lower potassium loss and a retention of water relative to substances producing a dilution of body fluid concentrations, as will be shown later by the data presented in Fig. 3. Taking the concentration of sodium in the extracellular fluids as 140 milli-equivalents per litre, the much larger body fluid loss in Experiment 3 as compared with Experiment 5 can be approximately accounted for by the loss of 16 milli-equivalents more of sodium in Experiment 3. Evidently the fluid loss in Experiment 5 is related chiefly to the loss of potassium which the administration of sodium chloride solution does not prevent.

Confirmation of this finding may be seen in Fig. 2 which presents the results of two diuretin experiments, one with and the other without the administration of saline, together with the two control experiments.² The experiments were on fasting animals over a 5 day period. In Experiments 6 and 7, diuretin was given on 4 days. In Experiment 7 each dose of diuretin was supplemented by 70 cc. of saline. The results are in agreement with those obtained in Experiments 3 and 5, Fig. 1. The fact that the data in Fig. 2 give slightly higher losses per day than those presented in Fig. 1 may be ascribed to the larger per day dose of diuretin and to the well established fact that losses per day in fasting decrease with time.

The behavior of several serum values in the presence of the losses of water and salt produced by diuretin was studied in two experiments, Nos. 8 and 4, and the results are presented in Fig. 3.

In the lower section of the figure the data describing the daily intake of water and the excretion of water, sodium, and chloride in the urine are recorded by means of pairs of columns, the total height of the left hand one measuring the water intake and that of the right hand one the volume of the urine. The relationship of the two values is thus easily seen. The measurements of excretion of sodium and of chloride are also laid off on these columns, sodium left and chloride right. In the upper part of the figure the measurements of daily nitrogen excretion in the urine and those of body weight are recorded, together with the several serum concentration values determined; *viz.*, protein, non-protein nitrogen, sodium, and chloride. The procedure in Experiment 8 consisted in giving a maize-fed animal five doses of diuretin dissolved in 100 cc. of distilled water in the course of a 9 day period of study at the intervals indicated at the bottom of the figure. In Experiment 4 the animal was fasted and received in the course of a 10 day period five doses of diuretin dissolved in 100 cc. of distilled water and in addition was given

² The controls are the first 5 days of the control Experiments 1 and 2, Fig. 1.

daily by stomach tube 150 to 200 cc. of distilled water in a single administration. The animals in both experiments had free access to water and the amounts taken were recorded.

From the results of Experiment 8 one observes a steady decline in weight and an approximately uniform relation between water intake and urine volume up to the last 3 hours of the experiment. In Ex-

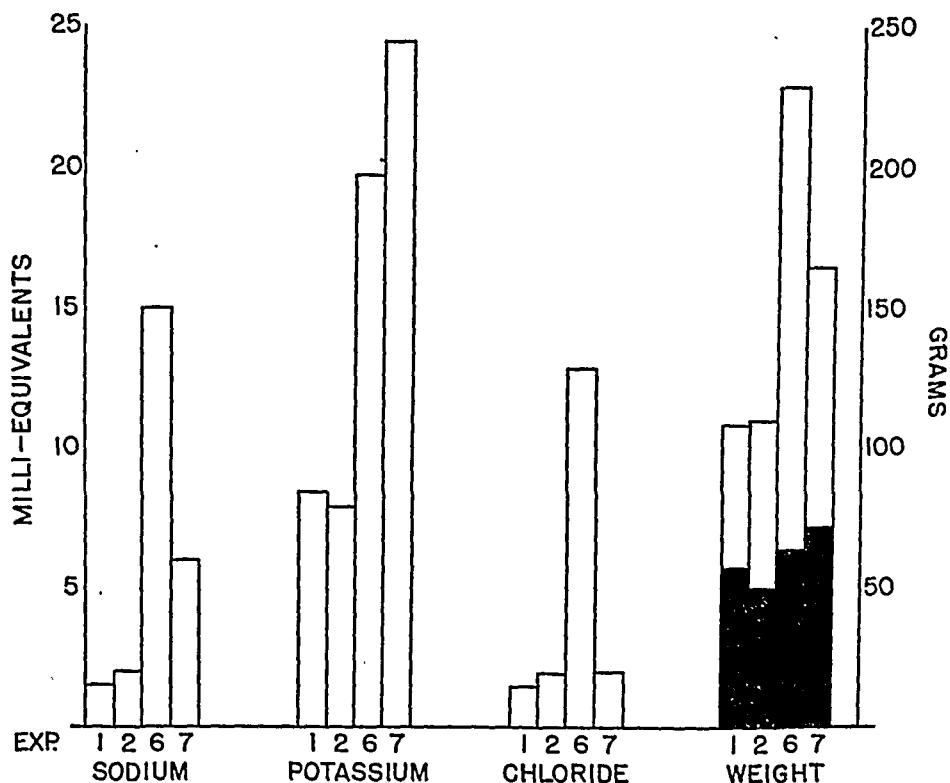


FIG. 2. Urinary excretions and weight losses, as described for Fig. 1, over a 5 day period. Experiments 1 and 2, fasting controls. Experiment 6, fasting rabbit receiving diuretin. Experiment 7, fasting rabbit receiving diuretin plus saline.

periment 4 there is a marked irregularity in the weight curve which is coincident with the marked irregularity in water intake-urine volume relationship. It is evident from the data that this results from the large difference between water intake and urine volume on the diuretin-free days and the large water diureses on the diuretin days. The data of the last day represent the 3 hours preceding death.

The serum concentrations found in Experiment 4, representing

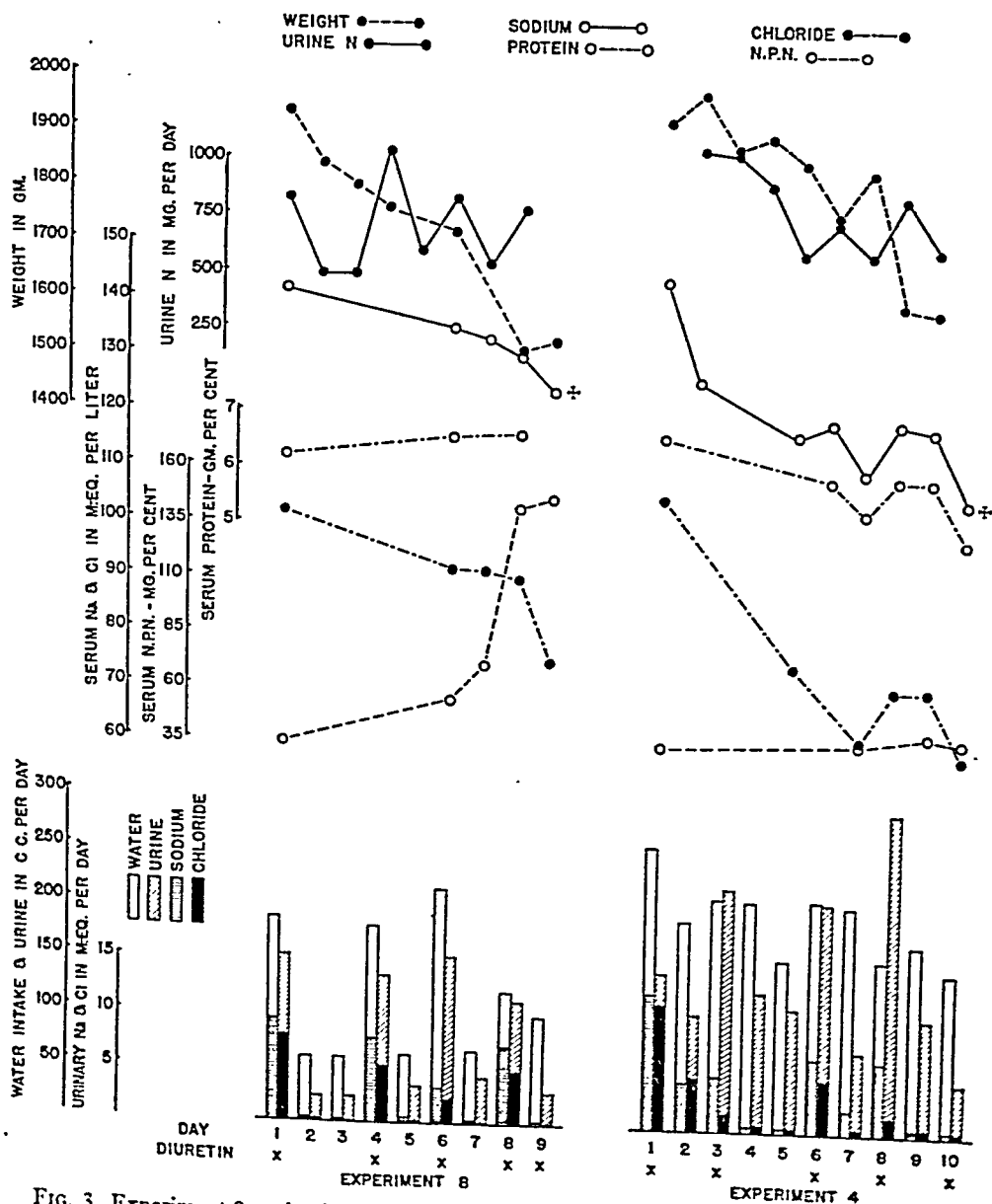


FIG. 3. Experiment 8, maize-fed rabbit receiving diuretin and water as desired. Experiment 4, fasting rabbit receiving diuretin, water as desired, and 150 to 200 cc. of additional water in single daily doses.

analyses of serum drawn 24 hours after the last administration of water, reflect the disturbance in water balance. For the first 5 days there was a large fall in serum concentrations as compared to those recorded from Experiment 8. The changes in serum sodium concentrations over the remainder of the experiment show an inverse relation between water retention and sodium concentration in spite of a large loss of sodium in the urine on the diuretin days. In so far as serum protein and chloride determinations are available, they confirm this effect of excessive water retention on serum concentrations. The fall in serum sodium and chloride concentrations on the last day of Experiment 8 reflects a similar influence of retained water. Until this last day of the experiment the loss of sodium and chloride incident to the diuretin is accompanied by water to such extent as results in but slight changes in serum concentration values as compared with those found in Experiment 4.

The failure of the total nitrogen excretion in Experiment 8 to increase in the presence of marked increase in serum N. P. N., we feel, reflects a diminution in the efficiency of kidney function as measured by nitrogen clearance. This deduction seems permissible on the basis of total nitrogen excretion, instead of the preferable non-protein nitrogen excretion, because it may be assumed that the protein nitrogen in the urine would tend to increase during the course of the experiment. Hence an error introduced by using total nitrogen excretion will increase and not decrease nitrogen clearance. Throughout the course of Experiment 4 the N. P. N. remained approximately constant and there is no significant divergence between nitrogen retention and excretion.

Thus the data from Experiments 8 and 4 contradict the existence of a direct relationship between hypochloremia and azotemia.

Table I presents data from other experiments which confirm the findings presented in Fig. 3. The data from Experiment 11 (Table I) particularly confirm the findings presented from Experiment 4 and discussed above. In this experiment, in which the animal was maize-fed, dehydration was minimal, as reflected by the small weight loss and excessive fall in serum concentrations, and no rise in N. P. N. occurred. The data further show that the degree of azotemia in the presence of similar dehydration is inversely proportional to the urine

volume; see Experiments 10, 3, and 6, Table I, and Experiment 8, Fig. 3. In other words the relationship of azotemia to the limitation of urine volume by dehydration is clearly indicated.

Table II presents data from the control experiments on fasting animals and fasting animals given diuretin and simultaneously salt solution. The spontaneous water intake of the fasting controls shows that the water given in the forced fluid experiments was not excessive, whereas the spontaneous water intake of the diuretin experiments without forced fluid was in the diuretin-free intervals rather low. No significant blood changes occurred either in the fasting controls or in

TABLE II
Average Daily Water Intake, Urine Volume, and Serum Concentrations at Beginning and End of Experiments

Experi- ment No.	Régime	Water intake	Urine volume	Na	Cl	HCO ₃	N.P.N.	Day of experi- ment
		<i>cc.</i>	<i>cc.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>	
1	Fasting	178	144	142	110	20	32	1
				137	98	19	49	9
2	Fasting	136	99	141	107	25	32	1
				138	95	23	30	9
5	Diuretin and salt	229	199	144	108	23	35	1
				140	100	23	35	9
7	Diuretin and salt	157	145	142	97		35	1
				138	99		30	5

the controls given diuretin and salt solution. The metabolism data of these animals are presented in Fig. 1, Experiments 1, 2, and 5, and Fig. 2, Experiments 1, 2, and 7.

DISCUSSION

The experiments reported here, as well as confirming the withdrawal of large amounts of chloride in the urine following the administration of diuretin, as reported by Gruenwald (1), also show a large withdrawal of sodium and potassium incident to diuretin administration. The urinary losses of sodium and chloride in our experiments

are equivalent to between three or four times the salt content of the initial plasma volume as estimated from Uthelm's data (17). These losses are approximately equal to the salt losses reported by Gamble and McIver (18) in rabbits dehydrated by pyloric obstruction. The average urinary potassium loss in the diuretin animals was double that in the fasting controls. Taking the potassium content of protoplasm from Goto's (19) analysis of muscle as 8 milli-equivalents per 100 gm., computation from the data represented by the potassium columns and the darkened portion of the weight columns in Figs. 1 and 2 shows that for a protoplasmic loss of 100 gm. there is an average loss of potassium corresponding in the control animals to 170 gm. and in the diuretin animals to 300 gm. of protoplasm, indicating in the diuretin experiments a much greater removal of potassium above that ascribable to tissue destruction. Such an event has been observed in fasting children by Gamble, Ross, and Tisdall (20) and in children with severe diarrhea by Butler, McKhann, and Gamble (21). Large losses of potassium have been reported following the use of acid-producing salts in rabbits by Goto (19) and moderate losses in human patients by Gamble, Blackfan, and Hamilton (22).

The sodium losses, described above, are accompanied in the experiments where the animals received water as desired by such losses of body water as to result in but small serum sodium dilutions. In other words, the tendency of the organism to maintain normal plasma base concentration, as emphasized by Gamble (23), is evident. In those animals given additional water in single large daily doses the sodium losses were not accompanied by such an excretion of water as to prevent large serum dilutions. Curtis (24) has shown that there is a delay in the onset of the diuresis following the administration of diuretics, if large amounts of water are given. And Darrow and Yannet (25) have observed in experiments of short duration low serum electrolyte concentrations and oliguria following the intraperitoneal administration of isotonic glucose solution which withdraws electrolytes into the peritoneal cavity and thus produces a fall of concentrations in the blood plasma. In our experiments over a period of several days the presence of serum dilution did not result in an excretion of water relative to salt to an extent sufficient to correct the low sodium and protein concentrations of the serum.

Comparison of the sodium and potassium losses above that ascribable to tissue loss which may be derived from the data shown in Figs. 1 and 2, with the dehydration portion of the corresponding weight losses, suggests not only the observed dilution of serum but also a dilution of tissue or intracellular potassium. The loss of sodium plus potassium in excess of the loss of water on the basis of their concentrations respectively in extracellular and intracellular fluids is particularly striking in Experiment 4, Fig. 1, where forced water was given with the diuretin and where a marked fall in serum concentrations was observed, Fig. 3.

TABLE III

Data Illustrating a Lack of Direct Relation between Serum Electrolyte Concentrations and Azotemia

Source	Total base	Na	Cl	N.P.N.	Δ
	m.-eq.	m.-eq.	m.-eq.	mg. per cent	°C.
Dog—withdrawal of pancreatic juice (29).....	160		94	200	
Withdrawal of gastric juice (8).....			69	34	
Same (8).....			53	196	
Dog—adrenalectomized (28).....	144	133	100	185	
Puppies—dehydrated by concentrated milk (26)...	236		160	192	0.95
Puppies—carbohydrate-rich, salt-free diet (30)....	120		67	15	0.41
Infants—diarrheal dehydration (27).....	156		104	74	0.57
Same (27).....	147		91	94	0.55
Rabbit—diuretin (present paper).....		130	90	143	
Same.....		101	62	42	0.42

The quantitative data with which a calculation of intracellular dilution in these experiments might be made are too rough to warrant an estimation. That such dilutions of intracellular potassium occur is confirmed by Goto's (19) figures, which show not only a withdrawal of muscle potassium from acid-fed rabbits but also a 20 to 30 per cent reduction in the potassium concentration of muscle.

Table III presents data from the literature (8, 30) confirming the lack of relation between azotemia and hypochloremia observed in our experiments. In dehydration coincident with the loss of approximately equivalent amounts of base and chloride, such as follows the loss of salt by withdrawal of pancreatic juice (29) or intestinal secre-

tions (27) or through the kidney, as in adrenal insufficiency (28) and in these diuretin experiments, azotemia may be present with but slight lowering of the serum chloride concentration. The observations of one of us (Kerpel-Fronius) in experiments on puppies dehydrated by feeding concentrated milk (26) illustrate an azotemia with dehydration in the presence of increased serum base and chloride concentrations. Coincident with the dehydration in his experiments there occurred an impairment in kidney function as evidenced by the fall in urine volume and urine nitrogen and chloride concentrations in the presence of increasing serum concentrations (31).

The ascribed dependence of azotemia on hypochloremia seems to have resulted from the frequency with which dehydration follows the loss of gastric secretions, in which the loss of chloride above the loss of base produces marked lowering of the serum chloride concentrations.

The data presented in our experiments together with those summarized in Table III indicate a consistent relation between azotemia and dehydration when of sufficient degree to greatly reduce urine volume. The effect of reduction of urine volume on nitrogen excretion is probably related to the dependence of urea clearance on urine volume as described by Van Slyke and coworkers (32). The *modus operandi* of dehydration in reducing nitrogen excretion, aside from a resulting oliguria, is not indicated by our experiments. Reasonably, however, nitrogen retention may be regarded as a result of other changes produced by dehydration such, for instance, as decrease in blood volume (Marriott (33) and McIntosh, Kajdi, and Meeker (34)), increase in blood viscosity (Surányi and Sonnauer (35) and observed qualitatively by us), and fall in blood pressure (Bottin (36) and Swingle *et al.* (37)), changes which together affect blood flow through the kidney and hence urine volume and urea excretion (Van Slyke (38)). From the evidence reported here the beneficial effect of salt solution in reducing the N. P. N. of non-nephritic azotemias is dependent upon an augmentation of urine volume rather than upon the restoration of serum electrolyte concentrations. The reduction of N. P. N. by administration of water without salt depends upon a temporary increase in urine and blood volumes at the possible risk of a hypotonicity of the blood which may approach that observed in water intoxication. Furthermore such water administration does not per-

manently remove the underlying cause of the nitrogen retention, namely the dehydration. It is incidentally interesting that the results of Experiment 4 demonstrate, in agreement with those of Darrow and Yannet (25), that the volume of the plasma may be defended at the expense of a fall in electrolyte concentrations in the presence of a presumably normal renal activity.

Another interesting point in these experiments is the clinical picture of the animals preceding death. The paralysis of the extremities, which extends from below upwards and ascends until death appears, is described in detail by Gruenwald (1). Having ruled out a toxic effect of the diuretin on the kidneys by histological examination and having been able to protect the animals from death by the simultaneous administration of salt with the diuretin, which removed the hypochloremia, Gruenwald was led to ascribe the final symptoms to hypochloremia. In our experiments death in the usual manner occurred after the fifth dose of diuretin even when the serum chloride concentration was but slightly below normal and it also occurred in the case of a fasting animal given salt with each dose of diuretin. Since we observed that the maize intake of salt-supplied animals was two to three times that of animals given diuretin without salt, we suggest that death in the last mentioned experiment was due to the marked loss of potassium which probably did not occur in Gruenwald's rabbits fed salt and maize.

SUMMARY

The losses of sodium, potassium, chloride, nitrogen, and water following the administration of diuretin to rabbits over 5 to 9 day periods together with the changes in serum concentrations of sodium, chloride, N. P. N., and total protein occurring simultaneously with these losses are described.

The circumstances responsible for the presence of azotemia in the animals were investigated in particular and the dependence of nitrogen retention upon dehydration and the modification of this dependence by variation in urine volume were demonstrated. It was clearly shown that no direct relationship exists between the azotemia and the coincident hypochloremia. It was found that nitrogen retention can be removed by the administration of water without salt, and the extent

to which serum electrolyte and protein concentrations can be lowered by this procedure was also observed.

The withdrawal from the body of large amounts of potassium as well as of sodium and chloride following the administration of diuretin, and also the inefficacy of sodium chloride solution in preventing the potassium loss was demonstrated.

We wish to acknowledge our indebtedness to Dr. James L. Gamble for advice throughout the work.

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THE EXPERIMENTAL PRODUCTION IN DOGS OF ACUTE
STOMATITIS, ASSOCIATED WITH LEUCOPENIA AND
A MATURATION DEFECT OF THE MYELOID
ELEMENTS OF THE BONE MARROW

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PLATES 5 AND 6

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The characteristic features of the rapidly progressive and frequently fatal disease of human beings known as acute agranulocytosis are leucopenia, granulopenia, stomatitis, and a suppression of maturation of the myeloid elements of the bone marrow. Many attempts have been made to reproduce the syndrome in animals, but although the granulopenia has been successfully simulated, an experimental condition presenting all of the features of the human disease has not been obtained.

In the course of a study of chronic black tongue in dogs (1) it was observed that in the animals dying of an acute form of the disease, pronounced leucopenia was frequently associated with the acute stomatitis. Moreover in certain instances the appearance of the oral lesions was strikingly similar to that seen in acute agranulocytosis of human beings. This observation prompted a more detailed study of the pathological changes of acute black tongue in order to ascertain whether more fundamental similarities existed.

Previous attempts to produce in animals a complex similar to acute agranulocytosis of man may be divided into two main groups: those employing bacteria, or their soluble products, and those depending upon the use of certain toxic chemical substances. Among the authors reporting experiments of the first type are Lovett (2), Piersol and Steinfeld (3), Fried and Dameshek (4), and Dennis (5) whose work failed of confirmation by Meyer and Thewlis (6). In spite of the very considerable number of experiments reported, the results have been somewhat inconstant and leucopenia associated with stomatitis has not been observed. The second group, in which toxic agents were used to cause agranulocytosis,

includes the work of Selling (7), Kline and Winternitz (8), Weiskotten (9), Turley (10), and Kracke (11). All depended upon the administration of some aromatic compound, in most instances benzol, but occasionally such substances as phenobarbital or amidopyrine. In these experiments again, the combination of stomatitis and leucopenia was not obtained, although a well defined suppression of maturation of the myeloid elements of the bone marrow was established.

The pathological changes in the bone marrow of human beings dying of acute agranulocytosis have been described by Schultz (12), Uffenorde (13), Dameshek (14), and Fitz-Hugh and his coworkers (15, 16). Widely varying degrees of cellularity have been reported but there is essential agreement that irrespective of the presence of hyperplasia or aplasia, the characteristic feature is a suppression of maturation of myeloid cells at some stage of their development.

Methods

Mongrel dogs weighing from 7 to 15 kilos were used. The diet fed was the modification of the Goldberger black tongue-producing diet described by Rhoads and Miller (1).

Articles of diet	Quantity	Nutrients		
		Protein	Fat	Carbohydrate
	gm.	gm.	gm.	gm.
Corn-meal.....	400	33.6	18.8	296
California black-eyed peas.....	50	10.7	0.7	30.4
Casein (purified).....	60	52		
Cane sugar.....	32			32
Cottonseed oil.....	15		30.0	
Cod liver oil.....	30		15.0	
Rice polishings.....	40			
Sodium chloride.....	3			
Calcium carbonate.....	10			
Total nutrients.....		96.3	64.5	358.4
Nutrients per 1,000 calories.....		40.1	26.9	149.3

Samples of blood were taken from the jugular vein at regular intervals, and the blood was collected in a standard amount of potassium oxalate to prevent coagulation. For counting the formed elements of the blood carefully calibrated pipettes and counting chambers were used. Smears for differential counts were stained by Wright's method.

Necropsies were done on all the animals. Specimens of bone marrow from the femur, tibia, rib, and sternum were fixed in Zenker's fluid with 5 per cent acetic acid as well as in Zenker's fluid containing 10 per cent formalin. The Zenker-

acetic acid-fixed tissue was stained with eosin-methylene blue while that fixed in Zenker-formol was stained by Giemsa's method. Similar procedures were employed in the study of other organs.

GENERAL RESULTS

Ten animals which developed an acute febrile disease characterized by stomatitis, leucopenia, granulopenia, and characteristic lesions of the bone marrow, are included in this report. In Table I is presented a summary of the essential findings. Four of the animals developed symptoms after more than 140 days of the experimental diet feeding, whereas less than 100 days sufficed in the case of the others. In two instances leucopenia accompanied the first attack of stomatitis, while in the others it did not occur until subsequent attacks. In all instances the leucopenia was observed together with, or soon after, the onset of the mouth lesions. No evidence is at hand to show that leucopenia preceded the stomatitis. Four of the dogs recovered from the first attack of leucopenia, and of these two died in a second attack, whereas one subsequently developed stomatitis which terminated in death without leucopenia. The fourth animal died of intercurrent disease without either stomatitis or blood dyscrasia.

Symptomatology.—The appearance of the stomatitis of acute black tongue has been described by Goldberger (17) and his coworkers and the pathological changes by Lillie (18).

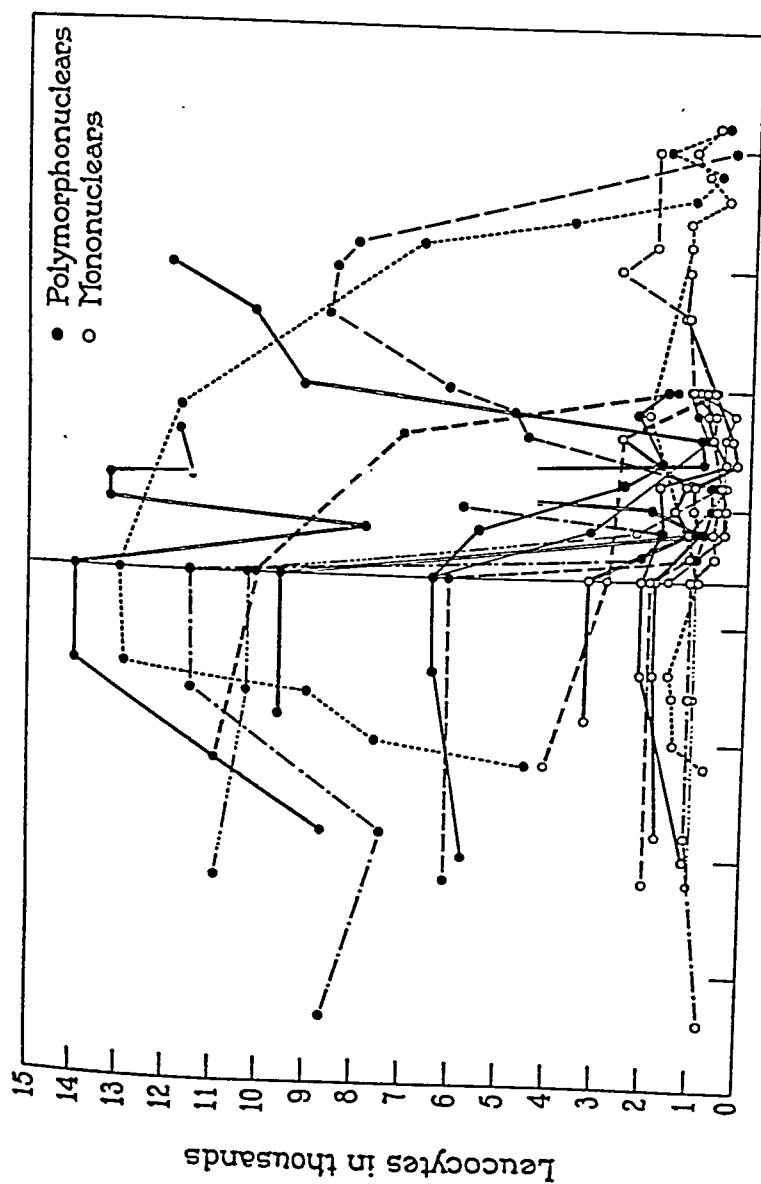
The earliest manifestation is a more or less extensive reddening of the mucosa of the lips, the floor of the mouth, or the cheeks. In the course of 12 to 24 hours the injection becomes a deep reddish purple color and soon after areas of superficial ulceration develop. Necrotic areas then form, composed of a grayish yellow center surrounded by a red margin. These areas extend rapidly and coalesce to involve the deeper layers of tissue with a change of the bright red color to a dirty yellow, with underlying deep purple, injected surfaces. A membrane of necrotic tissue, which can be scraped away easily, forms as a terminal feature. The odor is very fetid and salivation is marked.

In the studies here reported the tendency to the formation of deeply ulcerated, localized lesions was striking (Fig. 1). The necrosis was superficial at first but increased rapidly in extent to involve the deeper tissues. At the death of the animal gangrene had frequently occurred of a large part of the tonsillar fossa or floor of the mouth. Spiral and fusiform organisms morphologically similar to those found in ulcerative stomatitis of human beings were often present (Fig. 2). Masses of these organisms were injected into and under the gingival and buccal membranes of normal dogs without causing any perceptible reaction.

TABLE I

Summary of the Blood Findings before, during, and after Attacks of Induced Stomatitis with Leucopenia

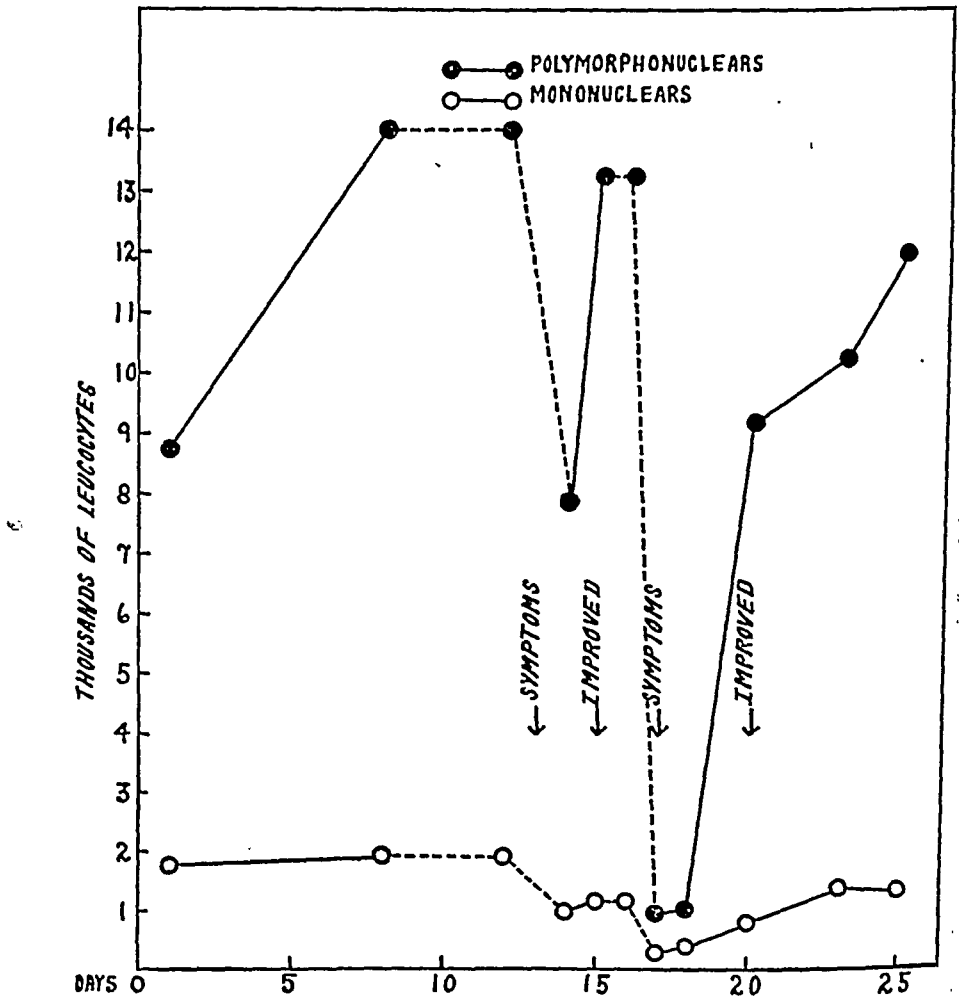
Dog No.	Length of time on diet	No. of attacks of stomatitis	Attack of stomatitis associated with leucopenia	Total W.B.C. before attack, per c.mm.	Absolute number of polymorphonuclear cells before attack, per c.mm.	Absolute number of mononuclear cells before attack, per c.mm.	Total W.B.C. during attack, per c.mm.	Absolute number of polymorphonuclear cells during attack, per c.mm.	Absolute number of mononuclear cells during attack, per c.mm.	Total W.B.C. after attack, per c.mm.	Remarks
	days										
1	85	2	2nd	8,100	6,460	1,640	1,350	830	520		Died
2	142	4	4th	9,800	7,150	2,650	1,800	1,120	680		Died
3	196	5	4th	8,150	6,050	2,100	1,250	750	500	9,300	Recovered
			5th	11,200	8,500	2,700	2,400	360	2,040		Died
4	101	2	1st	10,500	7,550	2,950	2,450	1,100	1,350	44,000	Recovered
											Died in subsequent attack without leucopenia
5	94	3	3rd	11,350	10,200	1,150	1,300	700	600		Died
6	79	2	1st	15,000	13,200	1,800	3,700	2,800	700	17,000	Recovered
			2nd	13,900	11,500	2,400	1,350	590	760		Died
7	97	3	3rd	12,600	11,500	1,100	2,500	1,900	600		Died
8	93	4	4th	7,300	5,500	1,750	2,350	1,750	550		Died
9	186	4	3rd	14,400	13,100	1,300	1,250	960	290	13,300	Recovered
											Died in subsequent attack without leucopenia
10	147	2	2nd	11,450	8,450	3,000	1,450	590	860		Died
Average...				11,146	9,100	2,045	1,927	1,135	791		



Five day intervals

TEXT-FIG. 1. Composite graph of the changes in polymorphonuclear and mononuclear leucocyte levels of ten dogs which developed acute black tongue. The perpendicular line indicates the time of onset of symptoms.

All of the animals were definitely ill from the time of onset of the stomatitis and refused food and fluid. The temperature was uniformly elevated and frequently reached 104–105°. Death usually occurred in from 2 to 3 days after the appearance of well defined disease manifestations.



TEXT-FIG. 2. Graph showing the change of leucocyte levels in a dog which developed acute black tongue with leucopenia and recovered.

Leucopenia.—A pronounced leucopenia with a marked decrease in the absolute number of polymorphonuclear cells was observed in all instances although in none was complete agranulocytosis present. In Table I are presented for all the animals the total and absolute numbers of polymorphonuclear and mononuclear cells present both before and during each attack of stomatitis and leucopenia. The lowest total leucocyte count observed during an attack was 1,250 per c. mm.,

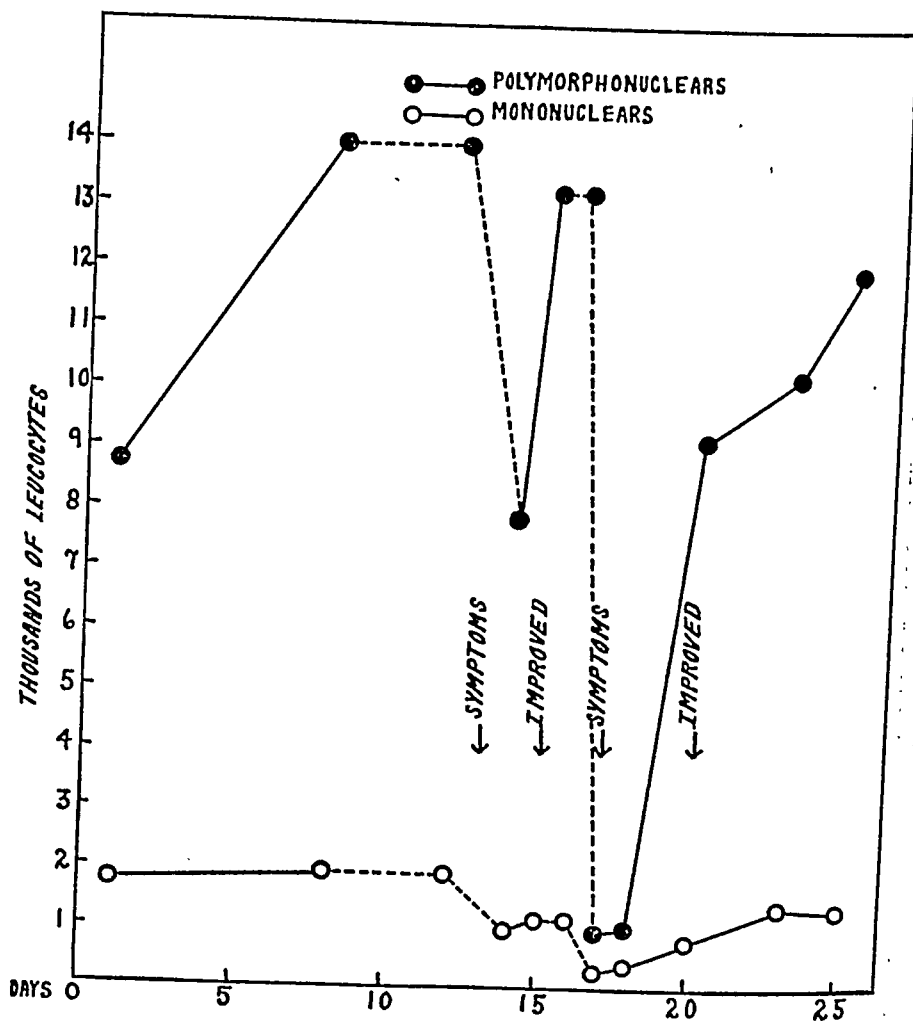
whereas the lowest absolute polymorphonuclear count observed was 360 per c. mm. The average total leucocyte count of all the animals before symptoms had occurred was 11,146 per c. mm. At the same time the average absolute polymorphonuclear count was 9,100 per c. mm. and the average absolute mononuclear count was 2,045 per c. mm. During the attacks of stomatitis and leucopenia the average total leucocyte count fell to 1,927 per c. mm., a decrease of 82 per cent. The average absolute polymorphonuclear count fell to 1,135 or a decrease of 90 per cent, while the average absolute mononuclear count fell to 791 per c. mm., or a decrease of 61 per cent. Of the four animals which recovered from attacks of leucopenia, one showed a total leucocyte count of 44,000 after recovery, a second of 17,000, and those of the remaining two dogs were not above normal.

Bone Marrow Alterations.—The femoral marrow of the normal dog is reddish yellow in color. Microscopically, islands of hematopoiesis (Fig. 3) are fairly widely separated by groups of fat cells. In the areas of hematopoiesis, erythrocytes, normoblasts, myelocytes, and polymorphonuclear cells with their various transition forms, bear a fairly constant numerical relation to each other. The normoblasts and the polymorphonuclear cells are the predominating cell types, but occasionally a small undifferentiated cell is seen. This cell has a small amount of cytoplasm which usually takes a mildly basophilic stain. The nucleus is round or slightly oval, contains a heavy chromatin network, and often one or more nucleoli. Sabin (19) considers this cell to be primitive in type and perhaps a precursor of both red and white blood cells.

A photomicrograph of a section of the bone marrow of a patient who died of acute agranulocytosis is presented (Fig. 4) which may be compared with the histological picture present in the marrows of the dogs which died with leucopenia and stomatitis (Figs. 5 and 6). In those animals the bone marrow shows a striking deviation from the normal. There are practically no adult polymorphonuclear cells, and few adult myelocytes. Most of the cells present are normoblasts and the so called primitive cells discussed above. The latter are present in far greater numbers than in the normal dog (Fig. 3) or human marrow (Fig. 4).

The degree of cellularity was not the same in all of the animal marrows; in some it was strikingly increased and in others it was even less than normal. However, the cell types were the same regardless of the cellular activity. No polymorphonuclear cells were seen; myelocytes and myeloblasts were not common; normoblasts and primitive cells predominated. In Fig. 6 primitive cells are present and certain forms suggest the transition of the primitive cell to the myeloblast. The maturation of erythropoietic cells also appears to have ceased, but the duration of the change was too short to be reflected in the peripheral blood. A striking feature was an intense vascular congestion, a change which is not uncommonly seen in the marrows of patients with acute agranulocytosis.

All of the animals were definitely ill from the time of onset of the stomatitis and refused food and fluid. The temperature was uniformly elevated and frequently reached 104–105°. Death usually occurred in from 2 to 3 days after the appearance of well defined disease manifestations.



TEXT-FIG. 2. Graph showing the change of leucocyte levels in a dog which developed acute black tongue with leucopenia and recovered.

Leucopenia.—A pronounced leucopenia with a marked decrease in the absolute number of polymorphonuclear cells was observed in all instances although in none was complete agranulocytosis present. In Table I are presented for all the animals the total and absolute numbers of polymorphonuclear and mononuclear cells present both before and during each attack of stomatitis and leucopenia. The lowest total leucocyte count observed during an attack was 1,250 per c. mm.,

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whereas the lowest absolute polymorphonuclear count observed was 360 per c. mm. The average total leucocyte count of all the animals before symptoms had occurred was 11,146 per c. mm. At the same time the average absolute polymorphonuclear count was 9,100 per c. mm. and the average absolute mononuclear count was 2,045 per c. mm. During the attacks of stomatitis and leucopenia the average total leucocyte count fell to 1,927 per c. mm., a decrease of 82 per cent. The average absolute polymorphonuclear count fell to 1,135 or a decrease of 90 per cent, while the average absolute mononuclear count fell to 791 per c. mm., or a decrease of 61 per cent. Of the four animals which recovered from attacks of leucopenia, one showed a total leucocyte count of 44,000 after recovery, a second of 17,000, and those of the remaining two dogs were not above normal.

Bone Marrow Alterations.—The femoral marrow of the normal dog is reddish yellow in color. Microscopically, islands of hematopoiesis (Fig. 3) are fairly widely separated by groups of fat cells. In the areas of hematopoiesis, erythrocytes, normoblasts, myelocytes, and polymorphonuclear cells with their various transition forms, bear a fairly constant numerical relation to each other. The normoblasts and the polymorphonuclear cells are the predominating cell types, but occasionally a small undifferentiated cell is seen. This cell has a small amount of cytoplasm which usually takes a mildly basophilic stain. The nucleus is round or slightly oval, contains a heavy chromatin network, and often one or more nucleoli. Sabin (19) considers this cell to be primitive in type and perhaps a precursor of both red and white blood cells.

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DISCUSSION

Our report deals with a group of experimental animals which died of acute black tongue following the feeding of a modification of the Goldberger black tongue-producing diet. The terminal features were an ulcerative, gangrenous stomatitis, in which spiral and fusiform organisms were found, leucopenia, granulopenia, and a suppression of maturation of the hematopoietic elements of the bone marrow.

The lesions of the mouth in acute black tongue have been described (18) and need not be discussed from a pathological viewpoint. The constant and persistent presence of spiral and fusiform organisms has suggested that they might be causative. Since the inoculation of masses of these organisms into and under the labial mucous membrane of normal dogs uniformly failed to produce lesions, it was concluded that they were secondary invaders. Lillie (18) has demonstrated lesions of the myelin of the nerves leading to the affected areas in canine black tongue, and hence it is assumed that the mucous membrane change is trophic in nature.

Two points concerning the leucopenia deserve special emphasis. Though pronounced in our animals, total or even almost complete absence of granulocytes was never observed. Secondly, in no instance did the sharp decrease in the number of circulating leucocytes precede the appearance of symptoms. It invariably followed or was coincidental with the earliest lesions of the mucous membranes. The leucopenia and oral lesions may be concomitant manifestations of a general disorder.

The histological changes of the bone marrow were characteristic. A similar dilatation of capillaries and cessation of maturation of hematopoietic cells have been observed in experimentally produced granulopenia following the administration of a number of toxic substances of the group of aromatic compounds. The marrow changes observed were clearly causal of the decrease of circulating leucocytes in the peripheral blood.

No explanation is at hand concerning the mode of action of the Goldberger black tongue-producing diet in causing suppression of hematopoiesis. The content of the diet in various dietary constituents has been discussed at length elsewhere (20) and need not be

detailed here. It suffices to state that the effects of feeding the diet may be prevented by the administration of a number of substances, among them meat and yeast. Conclusive evidence that the diet is lacking in some requisite dietary constituent capable of identification is not available. Pending further experiments, any speculation concerning the mode of action of the diet will be inconclusive at best.

SUMMARY

An ulcerative stomatitis associated with leucopenia and granulopenia can be induced in dogs by means of a diet causing black tongue. The decrease of circulating leucocytes is due to a suppression of maturation of the erythropoietic elements of the bone marrow. The changes as a whole have a resemblance to those occurring in human beings with acute agranulocytosis.

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EXPLANATION OF PLATES

PLATE 5

FIG. 1. Photomicrograph of a cross-section of an ulcerated lesion of the pharyngeal mucous membrane of a dog with acute stomatitis. Necrosis of the mucous membrane extending to the muscular layer is shown. Eosin-methylene blue. $\times 4$.

FIG. 2. Photomicrograph of the spiral and fusiform organisms present in the mucous membrane lesion shown in Fig. 1. $\times 1000$.

PLATE 6

FIG. 3. Photomicrograph of the femoral bone marrow of a normal dog. Both granulopoiesis and erythropoiesis are active. $\times 450$.

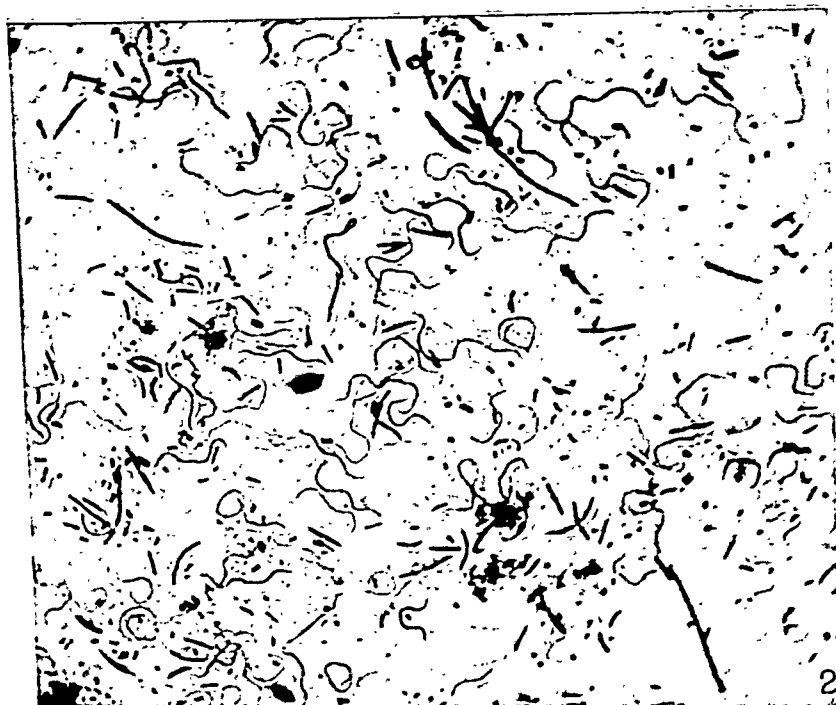
FIG. 4. Photomicrograph of the sternal bone marrow of a patient with acute stomatitis and granulopenia. Granulopoiesis is almost completely absent and erythropoiesis markedly reduced. $\times 450$.

FIG. 5. Photomicrograph of the femoral bone marrow of a dog with acute stomatitis and granulopenia. Both granulopoiesis and erythropoiesis are decreased. $\times 450$.

FIG. 6. Photomicrograph of the femoral bone marrow of a second dog with acute stomatitis and granulopenia. Changes similar to those in Fig. 4 are present. $\times 450$.



1



2

Photographed by Louis Schmidt

(Miller and Rhoads. Production of acute stomatitis)

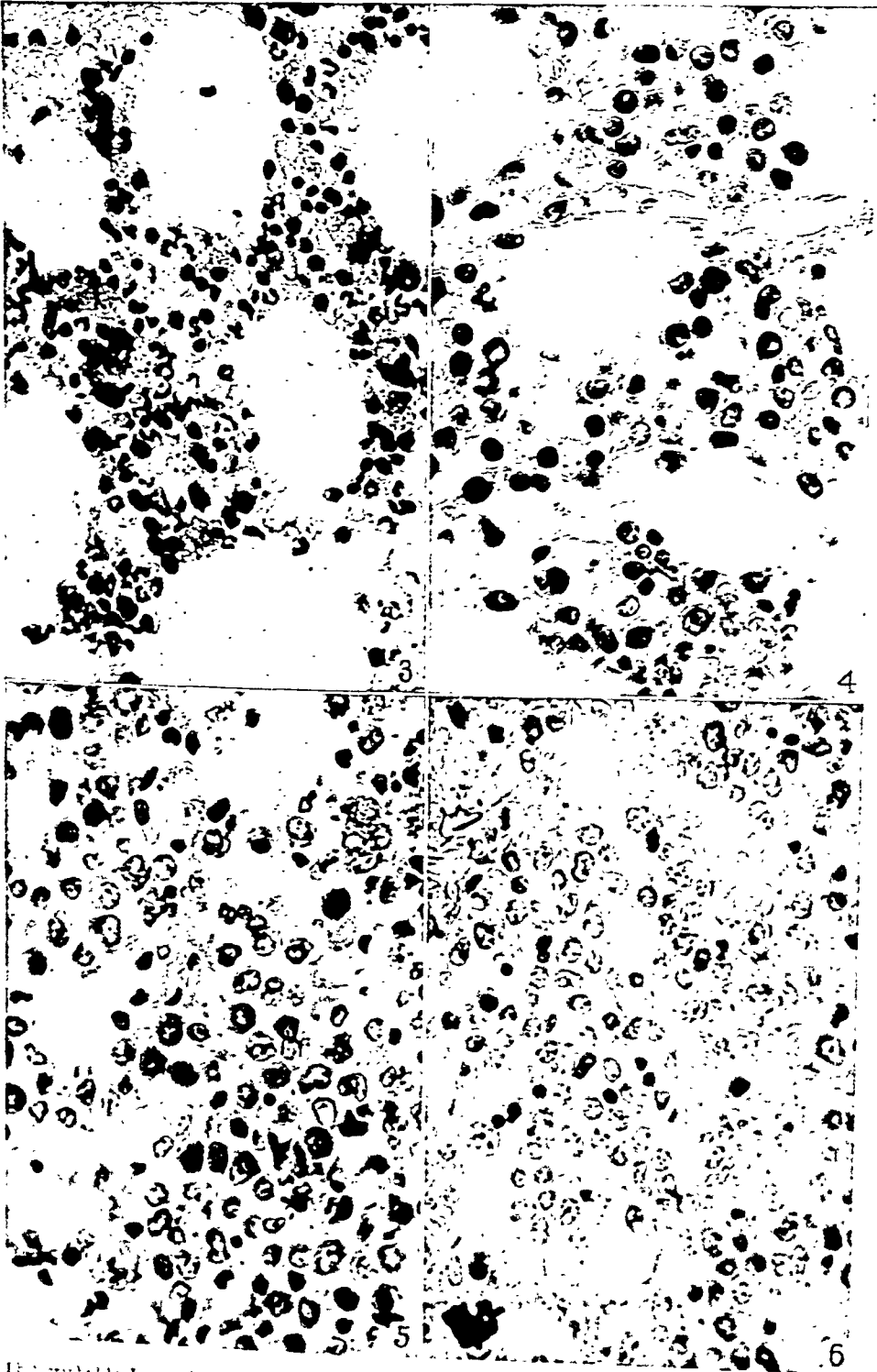


Fig. 3 and 5. Leucocytes.

Fig. 4 and 6. Erythrocytes.

FURTHER STUDIES ON KALA-AZAR

LEISHMANIA IN NASAL AND ORAL SECRETIONS OF PATIENTS AND THE BEARING OF THIS FINDING ON THE TRANSMISSION OF THE DISEASE

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Previous studies (1) of the nasal secretions of patients suffering from kala-azar yielded the following facts: (a) Smears from the nasal cavities of fifteen patients, when examined microscopically, revealed the presence of Leishman-Donovan bodies in nine cases. (b) Smears from the surface of the pharyngeal tonsil and from the saliva of one of these nine cases also showed the presence of leishmania. (c) The parenchyma of the tonsil of this latter case, at autopsy, was found to be massively infected with the parasites. (d) The nasal secretions of two patients were injected intraperitoneally into susceptible animals with the result that the animals became heavily infected, demonstrating that the parasites were viable.

These experiments showed for the first time that a rich source of infective material from a large proportion of patients with kala-azar was available for direct transmission of the disease. The present communication gives data confirming and extending the previous observations.

The medical literature contains almost no information concerning the presence or absence of leishmania in the secretions or tissues of the oral and upper respiratory tract of patients with kala-azar. The earliest study was that of Bentley (2) who in 1904 searched the sputum of patients, but failed to demonstrate the parasites. Carini (3) in 1911 and others subsequently found leishmania in the nasopharyngeal lesions of cases of espundia, a disease occurring in South America and caused by leishmania. This disease, however, has no similarity to kala-azar except that it is caused by a similar infectious agent. In 1913 Castellani (4) found leishmania in scrapings from chronic ulcers of the nasal mucous membrane of a case suspected of having kala-azar. Shortt (5) and his associates in 1932 examined various discharges and secretions of the body. They mentioned the saliva as a possible source of leishmania but did not examine it owing to the difficulty of using

cultural methods. The work of these latter investigators concerning leishmania in the urine and stools of patients was discussed in our previous paper (1).

Technical Details

The finding of Leishman-Donovan bodies in smears from the nasal secretions of patients with kala-azar is not difficult, but it demands careful preparation of the smears, a good microscope, an expert knowledge of the morphology of the parasite and persistence in searching. The parasites often are very few in number and may be scattered unevenly through the smear. Their small size and the presence in the nasal secretions of a variety of bacilli, cocci, cellular debris and albuminous material tend to render the parasites inconspicuous, and therefore prolong the time during which each oil immersion field must be examined before proceeding to the next.

We are convinced that the interested investigator can find the organisms in the smears without difficulty, but we are equally certain that search by the casual interne or the usual technician will be in vain. For the most part the smears on which these studies are based consisted of two or three preparations taken at one sitting only. A more vigorous search on several occasions might have yielded a higher percentage of positive findings.

There are several minor details in the preparation of the smears which are of value. An ordinary cotton-tipped culture swab was passed gently into the nasal cavity, and then onto the surface of a clean slide where the mucus and purulent material was spread thinly. These smears were allowed to dry in the air. Fixation with heat was avoided owing to its tendency to produce a finely divided precipitate of albuminous material. The slides were then flooded with Wright's stain and diluted with water in the manner ordinarily employed for blood smears. After washing, the smears were drained and allowed to dry in the air without blotting.

The animals used in these experiments were small Chinese field mice (*Cricetulus griscus*), commonly called hamsters, which were shown by Smyly and Young (6) to be highly susceptible to infection with *Leishmania donovani*. These animals, because of their cannibalistic tendencies, were kept in small, individual, wide meshed wire cages. In order to avoid cross-contamination among the animals and to render insect communication less likely, each cage containing either a control or an inoculated animal was suspended independently by a wire and separated from the others by an ample space of air. Under such conditions the hamsters survived and thrived for many months. Each animal was earmarked and in addition the cage was tagged with the animal number.

At times the intraperitoneal injections of saliva and nasal secretions were rendered difficult by the presence of thick mucus or crusts. Such material was prepared for injection by adding physiologic sodium chloride solution and then alternately filling and emptying a syringe onto which was attached at first large and then smaller needles. The feeding of infective material to the hamsters was accomplished by dropping the contents of a syringe into the open mouth and waiting for them to swallow while they were being held by an assistant.

Final examination of both control and inoculated animals was accomplished by autopsy. Smears were made of the spleen, liver and lymph nodes. These organs with other tissues were fixed in Zenker-formol (formalin 10 per cent) and after sectioning stained with hematoxylin and eosin.

The general plan was to inoculate four animals with each specimen of material. By such a procedure some allowance was made for accidental death of the animals and for individual differences in resistance and susceptibility to infection. Such a plan worked very well for the intraperitoneal injections of nasal secretions. However, the sputum or saliva of the patients when injected was much more likely to produce secondary pyogenic peritonitis which was fatal to a large proportion of the animals before sufficient time had elapsed for infection with leishmania to become manifest. As the result of these experiments it has been found that an interval of less than 45 days after intraperitoneal inoculation of either saliva or nasal secretions is insufficient for the infection with leishmania to be recognizable by the technique described. Hence animals which died earlier than the 45th day after inoculation were regarded as not surviving long enough for a satisfactory experiment. From our experience it would appear that the optimum time for the examination of animals after intraperitoneal inoculation with nasal secretions is about the 90th or 100th day, at which time heavy infection usually is present. Some animals show heavy infection as early as the 50th or 60th day.

EXPERIMENTAL

The nasal secretions of seven additional patients with kala-azar (Table I) have been examined microscopically. In three of these leishmania were present. This makes a total of twenty-two patients examined, in twelve of whom the parasites were found in the nasal secretions by direct examination. Smears from the tonsils of ten patients showed the parasites in three (Table V).

It will be noted that the nasal secretions of fourteen patients were inoculated intraperitoneally into hamsters (Table II). At the end of varying periods of from 45 to 200 days the animals were examined. From thirteen of the fourteen patients the nasal secretions were found to contain viable leishmania, evidenced by the infection of one or more of the hamsters of each group. The only patient in whom a negative result occurred was a small child from whom almost no nasal discharge could be obtained. Two animals only were inoculated from this patient and one of these did not survive long enough to be of value. There were three instances (Table V), Nos. P107, P115 and P103, in which direct examination of the nasal smears was negative but in which animal inoculation was positive.

Table III lists briefly the protocols of experiments concerning intra-peritoneal inoculations into Chinese hamsters of saliva, sputum and of material from pharyngeal tonsils of patients suffering from kala-azar. It will be noted that sputum or saliva or both from thirteen patients was inoculated into animals. In five of these cases the animals died

TABLE I

*Data concerning Patients with Kala-Azar in Whom the Nasal Contents Were Examined for Presence of Leishmania**

Case No.	Age	Sex	Duration of symptoms	Edge of spleen below left costal margin	State of the blood			Leishman-Donovan bodies in smears from		Globulin test on blood	Remarks
					R.B.C. per c.mm.	Hemoglobin per 100 cc.	W.B.C. per c.mm.	Spleen or liver	Nose		
	yrs.		mos.	cm.	mil-lions	gm.					
P109	3	M	8	11.2	2.86	6.2	2,500	Many	Neg.	++++	Died. Pneumonia
P110	14	M	12	8.0	3.90	10.9	2,750	Few	Pos.	+++	Recovery
P111	5	F	7	13.8	2.54	7.5	1,700	Moderate number	Pos.	+++	Recovery
P112	18	F	9	24.0	2.87	8.2	1,200	Moderate number	Pos.	++++	Recovery
P115	11	F	17	23.0	1.43	4.1	1,700	Moderate number	Neg.	++++	Recovery
P116	13	F	19	17.0	3.52	7.7	2,200	Moderate number	Neg.	++++	Recovery
P103	22	M	1	3.0	3.89	10.0	2,400	Very few	Neg.	Neg.	Recovery

* Leishman-Donovan bodies were found in nasal smears from nine of fifteen previously reported cases.

† These measurements were from the mid-clavicular line at the left costal margin to the tip of the spleen.

early in the course of the experiment, too soon for infection with leishmania to become discernible. In the other eight cases two, Nos. P108 and P111, showed leishmania to be viable in the saliva or sputum. In two other cases, Nos. P78 and P112 (Tables III and V), material from the tonsils, obtained in the first case at autopsy and in the second by puncture of the tonsil during life, when inoculated demonstrated the infectivity of the parasites.

TABLE II

*Data concerning Intraperitoneal Inoculation into Chinese Hamsters of Emulsions in Normal Saline of Nasal Secretions from Patients with Kala-Azar**

Case No.	Hamster No.	No. of animals	Amount inoculated	Duration of experiment	History of animals	Presence or absence of leishmania in sections or smears of			No. of hamsters surviving less than 45 days†	
						Spleen	Liver	Lymph nodes	Control	Inoculated
P94	6, 8	2	cc. 1	79-93	Killed	Pos.	Pos.	Pos.	4	2
	9	1	1	93	Killed	Neg.	Neg.	Neg.		
	7, 10, 11	3	(Control)	82-93	Killed	Neg.	Neg.	Neg.		
			None							
P78	14	1	1	45	Killed	Neg.	Neg.	Pos.	1	1
	15, 16, 17	3	1	62-118	Killed	Pos.	Pos.	Pos.		
	18, 19, 21	3	(Control)	48-109	Killed	Neg.	Neg.	Neg.		
			None							
P87	29, 30	2	0.25	81-104	Killed	Neg.	Neg.	Neg.	None	None
	31	1	0.60	180	Killed	Pos.	Pos.	Neg.		
	37	1	0.80	193	Killed	Neg.	Neg.	Neg.		
	36	1	(Control)	93	Found dead	Neg.	Neg.	Neg.		
			None							
P102	44	1	0.50	167	Killed	Pos.	Pos.	Neg.	None	3
	40	1	(Control)	189	Killed	Neg.	Neg.	Neg.		
P104	58, 59, 71, 72	4	0.20-0.25	111-186	Killed	Pos.	Pos.	Pos.	2	2
	60	1	0.20	69	Killed	Pos.	Pos.	Neg.		
	55, 61	2	(Control)	121-188	Killed	Neg.	Neg.	Neg.		
			None							
P105	78	1	0.20	106	Found dead	Pos.	Pos.	Neg.	None	None
	79, 80, 81	3	0.20	162-176	Killed	Pos.	Pos.	Pos.		
	97	1	(Control)	118	Killed	Neg.	Neg.	Neg.		
			None							
P107	108	1	0.20	99	Found dead	Pos.	Neg.	Neg.	None	None
	109	1	0.20	151	Killed	Neg.	Neg.	Neg.		
	110, 111	2	0.20	200	Killed	Pos.	Pos.	Pos.		
	112, 114	2	(Control)	48-87	Found dead	Neg.	Neg.	Neg.		
			None							

* Approximately equal quantities of nasal discharge and of normal salt solution were mixed together by means of drawing in and out of a syringe onto which was attached at first large and then smaller needles.

† 45 days was chosen arbitrarily as the minimum time after which a positive result could be expected in these experiments.

TABLE II—*Concluded*

Case No.	Hamster No.	No. of animals	Amount inoculated	Duration of experiment	History of animals	Presence or absence of leishmania in sections or smears of			No. of hamsters surviving less than 45 days†	
						Spleen	Liver	Lymph nodes	Control	Inoculated
P108	115, 117, 118	3	cc. 0.20–0.25	days 146–195	Killed	Pos.	Pos.	Pos.	None	None
	116	1	0.25	56	Found dead	Neg.	Neg.	Neg.		
	122	1	(Control) None	76	Found dead	Neg.	Neg.	Neg.		
P109	136	1	0.40	144	Killed	Neg.	Neg.	Neg.	None	1
	137	1	(Control) None	194	Killed	Neg.	Neg.	Neg.		
P110	123	1	0.25	146	Killed	Pos.	Pos.	Neg.	None	1
	124	1	0.25	195	Killed	Pos.	Neg.	Neg.		
	125, 126	2	0.20–0.25	195	Killed	Pos.	Pos.	Pos.		
	127	1	(Control) None	195	Killed	Neg.	Neg.	Neg.		
	144	1	(Control) None	113	Found dead	Neg.	Neg.	Neg.		
P111	145	1	0.25	143	Killed	Pos.	Pos.	Pos.	None	1
	147	1	0.30	112	Found dead	Pos.	Neg.	Pos.		
	152, 153	2	(Control) None	172	Killed	Neg.	Neg.	Neg.		
P112	169, 171	2	0.25	180	Killed	Pos.	Pos.	Pos.	None	None
	170	1	0.25	180	Killed	Neg.	Neg.	Neg.		
	172	1	0.25	101	Found dead	Neg.	Neg.	Neg.		
	162, 163	2	(Control) None	186	Killed	Neg.	Neg.	Neg.		
	164	1	(Control) None	123	Found dead	Neg.	Neg.	Neg.		
P115	197	1	0.30	173	Killed	Pos.	Pos.	Pos.	None	2
	199	1	0.30	113	Found dead	Pos.	Pos.	Neg.		
	205	1	(Control) None	173	Killed	Neg.	Neg.	Neg.		
P103	158, 159, 161	3	0.20	132–182	Killed	Neg.	Neg.	Neg.	None	None
	160	1	0.20	182	Killed	Pos.	Pos.	Pos.		
	155	1	(Control) None	182	Killed	Neg.	Neg.	Neg.		
	156, 157	2	(Control) None	150–169	Found dead	Neg.	Neg.	Neg.		

TABLE III

Data concerning Intraperitoneal Inoculations into Chinese Hamsters of Saliva, Sputum and of Material from Tonsils of Patients with Kala-Azar

Case No.	Hamster No.	No. of animals	Material inoculated	Amount inoculated	Duration of experiment	History of animal	Presence or absence of leishmania in sections or smears of		
							Spleen	Liver	Lymph nodes
P78	27, 28	2	Saliva	cc. 0.50	1 day	Found dead	—	—	—
	73	1	Scraping from cut surface of tonsil	0.20	59	Found dead	Pos.	Pos.	Pos.
	77	1		0.40	64	Killed	Pos.	Pos.	Pos.
	18, 19	2	None (control)	—	48-62	Killed	Neg.	Neg.	Neg.
P102	41	1	Saliva	0.25	19	Found dead	Neg.	Neg.	Neg.
	42	1		0.25	189	Killed	Neg.	Neg.	Neg.
P104	52, 53, 54	3	Saliva	0.25-0.35	155-209	Killed	Neg.	Neg.	Neg.
P105	100, 101, 103, 104	4	Saliva	0.20	17-137	Found dead	Neg.	Neg.	Neg.
P107	92, 93, 94	3	Saliva and sputum	0.30-0.40	1-6	Found dead	—	—	—
P108	119	1	Saliva	0.25	195	Killed	Pos.	Pos.	Pos.
	120	1		0.25	195	Killed	Pos.	Neg.	Neg.
	121	1		0.30	93	Found dead	Neg.	Neg.	Neg.
	122	1	None (control)	—	76	Found dead	Neg.	Neg.	Neg.
P109	133	1	Saliva	0.25	5	Found dead	—	—	—
	134	1		0.25	56	Found dead	Neg.	Neg.	Neg.
P110	138, 140	2	Saliva and sputum	0.25	194	Killed	Neg.	Neg.	Neg.
	141	1		0.50	76	Found dead	Neg.	Neg.	Neg.

TABLE III—*Concluded*

Case No.	Hamster No.	No. of animals	Material inoculated	Amount inoculated	Duration of experiment	History of animal	Presence or absence of leishmania in sections or smears of		
							Spleen	Liver	Lymph nodes
P111	148, 149, 150, 151	4	Saliva and sputum	cc. 0.30	days 137-172	Killed	Pos.	Pos.	Pos.
	152, 153	2	None (control)	—	172	Killed	Neg.	Neg.	Neg.
P112	165, 166, 167, 168	4	Saliva	0.25	1	Found dead	—	—	—
	193, 195	2	Abscess fluid from puncture of tonsil	0.20	1	Found dead	—	—	—
	194	1		0.10	175	Killed	Pos.	Pos.	Pos.
	162, 163	2	None (control)	—	186	Killed	Neg.	Neg.	Neg.
P115	200, 203	2	Saliva	0.40-0.50	7-9	Found dead	—	—	—
	201, 202	2		0.30	24-101	Found dead	Neg.	Neg.	Neg.
P116	208, 209	2	Saliva and sputum	0.40	1	Found dead	—	—	—
P103	47, 48	2	Saliva	0.25	10	Found dead	—	—	—

Having proved that resistant living leishmania were present in the nasal secretions of over 90 per cent of this small series of patients, it became of importance to know whether normal individuals could be infected easily when such secretions were administered by natural routes rather than by parenteral injections. Table IV gives brief protocols of a number of experiments in which nasal secretions, oral secretions and material from pharyngeal tonsils were introduced into the oral and nasal cavities of Chinese hamsters. This table also gives

data concerning inoculation of nasal secretions of patients with kala-azar into the nasal cavities of two normal human volunteers.

Perusal of the tables (Tables IV and V) reveals that the above experiments in many instances are still incomplete and in others that they have been only partially successful. Nasal secretions from the first case (No. P78) were inoculated into the nasal cavities of three animals. At the end of 59 days one of these animals (Hamster 22) was sacrificed. Search of the smears of the cervical lymph nodes and spleen revealed a few typical Leishman-Donovan bodies, but the infection was light and would have been missed had not a careful search been made. This experiment was of interest, but the slight degree of infection led to the prolongation of the time between inoculation and examination of the animals. The remaining two animals (Hamsters 23 and 24) together with two control hamsters (Nos. 25 and 26) were sacrificed after from 98 to 107 days. In none of these were leishmania demonstrable. However, three other animals (Hamsters 74, 75, 76) were fed with from 0.1 to 0.2 cc. of an emulsion in physiologic sodium chloride solution of material from the tonsil of the same patient. At the end of from 62 to 78 days all three were moderately heavily infected with leishmania.

Similar experiments with the nasal secretions and sputum of other patients, Nos. P87, P104, P105 and P107, are either incomplete or have been negative for transmission of the disease. Fifteen animals, Hamsters 128 to 131, 154, and 245 to 254, were the subjects of repeated feedings of nasal secretions from patients with kala-azar (Table IV). Four of these animals (Hamsters 129, 130, 154, 254), examined after from 97 to 107 days, were found to be uninfected with leishmania. One animal (Hamster 131) died too soon for the experiment to be of value. The remaining ten animals are still alive and will be examined at a later date.

Nasal secretions from two patients (Nos. P78 and P104) were inoculated (Tables IV and V) into the nasal cavities of two normal human volunteers (Nos. P67 and P72) on 3 successive days. These volunteers are well and show no signs of kala-azar 316 and 288 days respectively after the beginning of the experiment. Inasmuch as the incubation period of the disease in man is unknown, these volunteers will continue to be under observation for a considerable period of time.

TABLE IV

Data concerning Inoculation of Nasal and Oral Secretions and of Material from Tonsils of Patients with Kala-Azar into Oral and Nasal Cavities of Chinese Hamsters and Data concerning Inoculation of Nasal Secretions of Patients with Kala-Azar into Nasal Cavities of Two Human Volunteers

Case No.	Hamster or volunteer No.	No. of animals	Material inoculated	Amount inoculated	Route of inoculation	Duration of experiment	History of hamsters or of volunteers	Presence or absence of leishmania in sections or smears of		
								Spleen	Lymph	Lymph nodes
P78	22	1	Nasal secretion	Few drops	Nasal	days	Found	Pos.	Neg.	Pos.
	23, 24	2				59	dead	Neg.	Neg.	Neg.
	74	1	Scraping from cut surface of tonsil	0.10 cc.	Oral	64	Killed	Pos.	Neg.	Neg.
	75	1		0.20 cc.		62	Found	Pos.	Pos.	Pos.
	76	1		0.20 cc.		78	dead	Pos.	Pos.	Pos.
	P67 (volunteer)	1	Nasal secretion	Few drops	Nasal	Incomplete	Killed	—	—	—
P87	25, 26	2	None (control)	—	—	107	Killed	Neg.	Neg.	Neg.
	32, 33	2	Nasal secretion	0.30-0.40 cc.	Oral	104-138	Killed	Neg.	Neg.	Neg.
	34	1				Incomplete	Living	—	—	—
	35	1	None (control)	—	—	Incomplete	Living	—	—	—
	36	1				93	Found	Neg.	Neg.	Neg.
							dead			

P101	49, 50 51	2 1	Nasal secretion	5-6 drops 7 drops	Oral Oral and nasal	Incomplete 119	Living Killed	— Neg.	— Neg.
	63 64, 65, 66	1 3		0.20 cc. 0.10-0.20 cc.	Oral Oral	Incomplete 101-170	Living Found dead	— Neg.	— Neg.
	67	1		0.20 cc.	Oral and nasal	Incomplete	Living	—	—
	P72 (volunteer)	1		0.45 cc.	Nasal	Incomplete	Well	—	—
	55, 61 69, 70	2 2		— —	— —	121-188 Incomplete	Killed Living	Neg. —	Neg. —
P105	88, 90, 91 89a	3 1	Nasal secretion	0.15-0.20 cc.	Oral	Incomplete 173	Living Killed	— Neg.	— Neg.
	97 98	1 1		— —	— —	118 234	Killed Found dead	Neg. Neg.	Neg. Neg.
	105	1		—	—	Incomplete	Living	—	—
	95 96	1 1		3 drops 4 drops	Oral Oral and nasal	Incomplete Incomplete	Living Living	— —	— —
	106, 107 113	2 1		Few drops —	Oral —	Incomplete Incomplete	Living Living	— —	— —
P107			Sputum						

TABLE IV—*Concluded*

Case No.	Hamster or volunteer No.	No. of animals	Material inoculated	Amount inoculated	Route of inocu- lation	Duration of experiment days	History of hamsters or of volunteers	Presence or absence of leishmania in sections or smears of		
								Spleen	Liver	Lymph nodes
P108; P110; P103; P111; P112; P115; P116*	128	1	Nasal secretion: P108 (2X); P110; P103 (2X); P112 (3X); P115; P116 Sputum: P111	Few drops at each re- peated inoculation	Oral and nasal	Incomplete	Living	—	—	—
P108; P110; P103; P110; P111; P112; P115; P116	129, 130	2	Nasal secretion: P108 (2X); P110; P103 (2X); P112 (3X); P115; P116 Sputum: P110; P111	Few drops at each re- peated inoculation	Oral and nasal	97-107	Killed	Neg.	Neg.	Neg.
P108; P110; P111	131	1	Nasal secretion: P108 (2X); P110 Sputum: P111	Few drops at each re- peated inoculation	Oral and nasal	7	Found dead	—	—	—
P103; P112; P115; P116	154	1	Nasal secretion: P103; P112 (3X); P115; P116	Few drops at each re- peated inoculation	Oral and nasal	98	Found dead	Neg.	Neg.	Neg.

P103; P110; P103; P111; P112; P110; P115; P116	132	1	None: Control for Hamsters 128, 129, 130, 131	—	—	164	Found dead	Neg.	Neg.
P119; P120; P140; P141; P142; P143	245-253 254	9 1	Nasal secretions; P119; P120; P140; P141; P142; P143	Few drops at each re- peated inoculation	Oral and nasal	Incomplete 103	Living Found dead	— Neg.	— Neg.

* The studies tabulated in this and the subsequent horizontal columns were concerned with repeated inoculations from a series of patients with kala-azar.

TABLE V
Summary

Results of Microscopic Examination, of Intraperitoneal and Oral and Nasal Inoculation into Chinese Hamsters and of Nasal Inoculation into Human Volunteers of Material from Patients with Kala-Azar

Case No.	Microscopic examination			Results of intraperitoneal inoculation into hamsters of			Results of inoculation into nasal and oral cavities of hamsters of			Results of inoculation of nasal discharge into nasal cavities of human volunteers
	Material from liver or spleen puncture	Smears from nasal cavity	Smears from tonsils	Nasal discharge	Sputum or saliva	Material from tonsils	Nasal discharge	Sputum	Material from tonsils	
P94	Pos.	Pos.	—	Pos.	—	—	—	—	—	—
P95	Pos.	Neg.	—	—	—	—	—	—	—	—
P96	Pos.	Neg.	—	—	—	—	—	—	—	—
P97	Pos.	Pos.	—	—	—	—	—	—	—	—
P98	Pos.	Neg.	—	—	—	—	—	—	—	—
P78	Pos.	Pos.	Pos.	Pos.	Animals died too soon*	Pos.	Pos.	—	Pos.	Incomplete
P84	Pos.	Neg.	—	—	—	—	—	—	—	—
P99	Pos.	Pos.	—	—	—	—	—	—	—	—
P87	Pos.	Pos.	—	Pos.	—	—	Incomplete	—	—	—
P89	Pos.	Neg.	—	—	—	—	—	—	—	—
P102	Pos.	Pos.	Neg.	Pos.	Neg.	—	—	—	—	—
P104	Pos.	Pos.	Neg.	Pos.	Neg.	—	Incomplete	—	—	Incomplete
P105	Pos.	Pos.	Pos.	Pos.	Neg.	—	Incomplete	—	—	—
P107	Pos.	Neg.	—	Pos.	Animals died too soon*	—	Incomplete	Incomplete	—	—
P108	Pos.	Pos.	Neg.	Pos.	Pos.	—	—	—	—	—
P109	Pos.	Neg.	Neg.	Neg.	Neg.	—	—	—	—	—
P110	Pos.	Pos.	Neg.	Pos.	Neg.	—	—	—	—	—
P111	Pos.	Pos.	Neg.	Pos.	Pos.	—	—	—	—	—

P112	Pos.	Pos.	Pos.†	Pos.	Animals died too soon*	Pos.	—	—	—	—
P115	Pos.	Neg.	Neg.	Pos.	Animals died too soon*	Pos.	—	—	—	—
P116	Pos.	Neg.	—	Pos.	Animals died too soon*	Pos.	—	—	—	—
P103	Pos.	Neg.	—	Pos.	Animals died too soon*	Pos.	—	—	—	—
Total..22	22 pos.	12 pos. 10 neg.	3 pos. 7 neg.	13 pos. 1 neg.	2 pos. 6 neg.	2 pos.	1 pos. 4 incomplete	1 incomplete	1 pos.	2 incomplete

* 45 days was chosen arbitrarily as the minimum time after which a positive result could be expected in these experiments.

† This positive smear was from material obtained by puncture of the tonsil.

DISCUSSION

The subject of the natural mode or modes of transmission of kala-azar has been one of greatest importance since the nature of the disease was first clearly recognized by Leishman (7) and by Donovan (8) in 1903. There exist large areas of heavily populated districts in China, India and in the countries bordering on the Mediterranean Sea where the disease is prevalent, the mortality high and facilities for treatment exceedingly few. One of the most hopeful avenues for escape from the ravages of the disease would be to discover its mode of transmission and thereby aid in the eradication of the conditions favoring its spread. To solve this problem has been the chief aim of various kala-azar commissions, and in addition to them many independent investigators have pursued the problem in its various aspects. Chief among the theories has been that the sand-fly actively transmits both kala-azar and oriental sore in much the same manner as trypanosomiasis, etiologically a somewhat closely related disease, is transmitted by the tsetse fly. Hundreds of papers and scores of investigators have dealt with this aspect of the problem, attempting by every conceivable means to incriminate the sand-fly. Strong presumptive evidence supports this theory but in spite of exhaustive attempts it has not yet been shown that the sand-fly is an important agent in the transmission of the disease. Out of many hundreds of susceptible animals, exposed each one to scores or in some instances hundreds of bites of infected sand-flies, in only three instances (9, 10) has kala-azar and in one instance (11) oriental sore been thought to have been transmitted. Eleven human volunteers subjected themselves to hundreds of bites of sand-flies known to be infected but there resulted no transmission of the disease. In fact the large volume of excellent work on the subject gives strong testimony that the sand-fly is not an important agent in the transmission of the disease. It seems conceivable that some insect other than the sand-fly or even the sand-fly itself may be shown eventually to be an important intermediate host. The chief evidence for and against the transmission of leishmaniasis by the bite of the sand-fly as accumulated from the voluminous literature on the subject, is listed in Table VI.

The second theory, that of transmission by direct or indirect contact

TABLE VI

Facts for and against Transmission of Leishmaniasis by Bite of Sand-fly

For	Against
1. Sand-flies readily become infected by feeding on oriental sores or on patients or animals with kala-azar	1. Rare to find infected sand-flies even in the houses of kala-azar patients
2. Occasional sand-flies caught in nature have been infected with leishmania	2. Sand-flies usually feed only once on mammalian hosts, but may with difficulty be forced to feed a second or third time. It is only after the second or subsequent feedings that the buccal cavity contains leishmania
3. Correlation exists between site on the body of bites of sand-flies and of occurrence of oriental sores	3. Cases of kala-azar have occurred in individuals who have never been in contact with sand-flies
4. Skin of infected individuals may be heavily infected with leishmania	4. Sand-flies exist in many parts of the world where kala-azar is not known to occur
5. Wherever oriental sore or kala-azar is endemic sand-flies have been found	5. Cultures of skin where infected sand-flies had been feeding were negative for leishmania
6. Leishmania ingested by sand-fly undergo development into flagellates and invade the buccal cavity	6. Out of many hundreds of susceptible animals exposed each to numerous bites of infected sand-flies in only three instances has kala-azar and in one instance has oriental sore been thought to have been transmitted
7. Infected sand-flies when fed through a membrane on sterile fluid produce infection of the fluid with leishmania	7. No instance of transmission of disease resulted when eleven human volunteers were subjected to hundreds of bites of infected sand-flies
8. Inoculation into skin of man and dog of emulsion of infected sand-flies produced dermal leishmaniasis	8. When infected and normal animals have been kept in the same cage, free from ectoparasites, infection of the healthy animals has resulted
9. Three hamsters subjected to numerous bites of infected sand-flies over prolonged periods of time acquired leishmaniasis after an interval of over 400 days	
10. One healthy dog in the same cage with sand-flies and an infected dog acquired the disease	

TABLE VII

Facts for and against Transmission of Leishmaniasis by Direct or Indirect Contact

For	Against
1. Viable parasites have been found in intestinal and gastric mucosa and in urine and stools of infected individuals	1. <i>Leishmania</i> ordinarily do not go through their complete life cycle (flagellation) at 37°C. or in the body of man
2. Kala-azar is notorious as a family and a house disease	2. Only one of thirty-two hamsters fed 151 times with feces of infected hamsters acquired the disease
3. During epidemic periods spread of disease required intimate contact	3. One monkey fed repeatedly on feces of a patient with kala-azar failed to become infected
4. Presence of infected and healthy animals in same cage results in infection of the normal animal	4. None of thirty-two hamsters fed repeatedly on deposit from fresh centrifuged urine of kala-azar cases, acquired the disease
5. Susceptible animals readily acquire disease if fed infected material (organs, cultures, infected sand-flies or ticks)	5. Many cases of endemic kala-azar arise in which there is no history of contacts with infected individuals. It is well known, however, that heavily infected individuals may remain symptom-free at least for many months
6. One case in man presumably infected by accidental sucking into the mouth of infected material	6. Kala-azar, at least in many districts, appears to be a rural disease, many more cases coming from the outlying districts of villages than from within the confines of the village itself
7. The form of leishmania found in bodies of infected patients can produce infections when administered orally to animals	7. No rich source of infective material from patients with kala-azar has been demonstrated
8. Flagellation is not a necessary phase for the reproduction of the parasite or for its infectivity	
9. Some cases of kala-azar cannot be explained satisfactorily on the basis of insect transmission	
10. House-flies and dog fleas readily ingest and may 5 minutes later deposit in their dejecta living leishmania	
11. Parasites remain viable and may multiply in milk for periods of days or months	

TABLE VII—*Concluded*

For	Against
12. <i>Leishmania</i> may survive or even may be cultivated in presence of bacterial infection with cocci and bacilli	
13. <i>Leishmania</i> survive after marked changes have occurred in their chemical or physical environment	

infection, has fewer adherents and has been tested much less rigorously. The major points in the evidence both for and against this mode of transmission are listed in Table VII. One of the chief arguments against this theory has been that no adequate source of infective material from patients with kala-azar has been demonstrated.

The results of the experiments here reported remove this objection in that it has been demonstrated that patients, whether examined early or late in the disease, almost without exception discharge in their oral and nasal secretions viable and infective parasites. In some instances, Nos. P107, P115 and P103 (Table V), direct examination of the nasal secretions was negative, but animal inoculation was positive. Such findings indicate that although the number of organisms injected must have been exceedingly few, yet their pathogenicity was such that massive infection occurred. Furthermore, these experiments show that the Leishman-Donovan body exists and retains unaltered its infectivity even in the presence of various and sundry types of bacteria which in number greatly exceed the leishmania. Such facts demonstrate a degree of resistance of the parasite which has been too little appreciated.

Although we have succeeded in transmitting the disease by means of introducing nasal secretions from patients into the nose and mouth of a susceptible animal and by means of transferring as little as 0.1 cc. of an emulsion of material from the pharyngeal tonsil into the mouth of another susceptible animal, yet the results have not been successful uniformly. These studies have by no means demonstrated that the usual mode of transmission of kala-azar is through the agency of direct contact, but they have added new facts to knowledge of the disease which lend additional weight to such an hypothesis.

SUMMARY

The summary of our experiments (Table V) including data previously reported is as follows:

1. Twenty-two patients were studied, in all of whom the diagnosis of kala-azar was confirmed by puncture of the spleen or liver, with recovery of Leishman-Donovan bodies.

2. Microscopic examination of the nasal secretions revealed typical parasites in twelve (54.5 per cent) of the twenty-two cases.

3. Microscopic examination of smears from the pharyngeal tonsils of ten patients showed parasites in three (30 per cent).

4. The results of intraperitoneal inoculation into hamsters of nasal discharge from fourteen patients proved that the parasites were present and had retained their infectivity in thirteen (92.8 per cent).

5. Intraperitoneal inoculation into animals of sputum or saliva killed many animals before sufficient time had elapsed for the presence or absence of infection with leishmania to be demonstrated. However, in eight cases animals survived and in two of these (25 per cent) transmission of the disease had occurred.

6. Material obtained from the pharyngeal tonsils of two patients, when inoculated intraperitoneally into hamsters resulted in infection with leishmania in both instances.

7. Single inoculations, into the oral and nasal cavities of hamsters, of nasal discharge from five patients have resulted in transmission of leishmaniasis in one instance only, but these experiments are incomplete.

8. Repeated inoculations by the oral and nasal routes of hamsters and of two human volunteers with nasal secretions from patients with kala-azar have not yet been concluded, but to the present date have resulted negatively.

9. Emulsions of material from the pharyngeal tonsil of one patient, when fed to three hamsters in amounts of from 0.1 to 0.2 cc. on one occasion only, resulted in generalized infection with leishmania in each of the animals.

10. Evidence as obtained from the medical literature both for and against the transmission of kala-azar by direct or indirect contact and by means of the bite of the sand-fly is presented.

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CONCLUSIONS

1. Patients with kala-azar, whether the symptoms of the disease are of short or of long duration, almost without exception have present in their oral and nasal discharges viable, pathogenic *Leishmania donovani*.
2. Evidence is presented which strongly supports a theory of transmission of kala-azar by means of direct or indirect contact infection.
3. Unequivocal proof of the important natural mode or modes of transmission of kala-azar has not yet been presented. Much more work must be done before a final solution to the problem can be accepted.

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DIAGNOSIS OF PSITTACOSIS IN MAN BY MEANS OF INJECTIONS OF SPUTUM INTO WHITE MICE

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Since the pandemic of psittacosis in 1929-30, instances of disease in human beings associated with parrots and parakeets have continued to appear, either as isolated occurrences or as small localized epidemics. Frequently the malady has manifested itself by unusual clinical pictures in the patients and has run a course not usually considered to be characteristic of psittacosis. Furthermore, at times the disease has occurred in people associated with birds which have apparently been in good health in this country for considerable periods of time and which until recently would have been considered "safe" in the sense of being free from psittacosis.

Bedson (1) and his coworkers demonstrated the virus of psittacosis in the blood of patients suspected of having the disease by injecting samples of their blood into budgerigars. When we began our studies on psittacosis in 1930 we immediately recognized the fact that it would not be safe to use budgerigars or other small members of the psittacine family for the diagnosis of the disease in human beings, (1) because such birds, the natural hosts of psittacosis, might be carriers of the virus, and (2) because it is exceedingly dangerous to have live birds that are infected with psittacosis virus in the laboratory or in the animal house.

Five weeks after we began to study psittacosis one of the doctors in the laboratory became sick and it was thought that he had accidentally contracted the disease. Being aware of Krumwiede's (2) experiments that showed white mice are susceptible to the virus of psittacosis, and suspecting that the virus might be in the sputum of an individual sick of psittacosis, inasmuch as the malady exhibits itself as a pneumonia, we injected small amounts of sputum from the patient into the

peritoneal cavities of 6 mice. In this manner we recovered virus from the sputum of the patient (3) and confirmed the clinical diagnosis of psittacosis.

A reasonably safe laboratory method for the diagnosis of psittacosis in man is of importance. Our investigations (4), as well as similar experiences of others, have indicated that serological tests are probably not suitable for the detection of psittacosis. Following Krumwiede's observations, our work (5) and the findings of others have shown that mice are highly susceptible to psittacosis and that the experimental disease in them can be easily recognized. We have found, furthermore, that mice can be used for the diagnosis of psittacosis in man by means of injections of washed sputum or filtrates of sputum into their peritoneal cavities, and that infected mice can be handled with a minimum of danger of accidental infection. The work, already presented in a preliminary note (6), will now be given in greater detail.

Sputum-Mouse Test for Diagnosis of Psittacosis

In the test for the presence of virus in the sputum of human beings suspected of having psittacosis either unfiltered or filtered sputum may be used. The test is conducted in the following manner.

Unfiltered Sputum.—Material coughed up from the lungs—not saliva or discharges from the nasopharynx—is washed and then emulsified in physiological salt solution or Locke's solution by means of repeated passages through a 20 gauge needle attached to a syringe. Six white mice are inoculated intraperitoneally with the emulsion, 3 receiving 0.25 cc. each, while 3 others get 0.5 cc. each. Too rich an emulsion should not be used, because the mice must be able to destroy the bacteria in order that the virus may be obtained free from contaminants. The animals are observed for a period of 30 days.

Filtered Sputum.—The sputum of the majority of patients with psittacosis does not contain organisms, such as pneumococci and hemolytic streptococci, sufficiently virulent to kill the mice, and under these conditions it is not necessary to filter the sputum, provided too large an inoculum is not used. At times, however, the bacteria in the sputum are so virulent that it is impossible to test for psittacosis virus in their presence. Then it is essential to filter the sputum and inoculate mice with the filtrate in the following manner.

The patient's sputum to which 20–50 volumes of meat infusion broth, pH 7.8, and a small amount of alundum have been added is thoroughly ground in a mortar. The emulsion is centrifuged for 10 minutes at a speed of 3000 R.P.M. Then the supernatant fluid is filtered through a Berkefeld V candle at a pressure of 15–30

cm. of mercury. Each of 6 mice receives intraperitoneally¹ on 3 successive days 2 cc. of the filtrate. The animals are observed 30 days.

Housing of Mice during the Test.—The mice are housed in battery jars, 6 mice being put in a jar. The jars are placed in large flat trays containing a shallow layer of a 5 per cent solution of lysol. The trays are then set on tables the legs of which stand in basins filled with a 5 per cent solution of lysol (Fig. 1), and the tables are kept in a well screened room. These precautions are taken to prevent the mechanical spread of infection by insects. Inasmuch as some mice have to

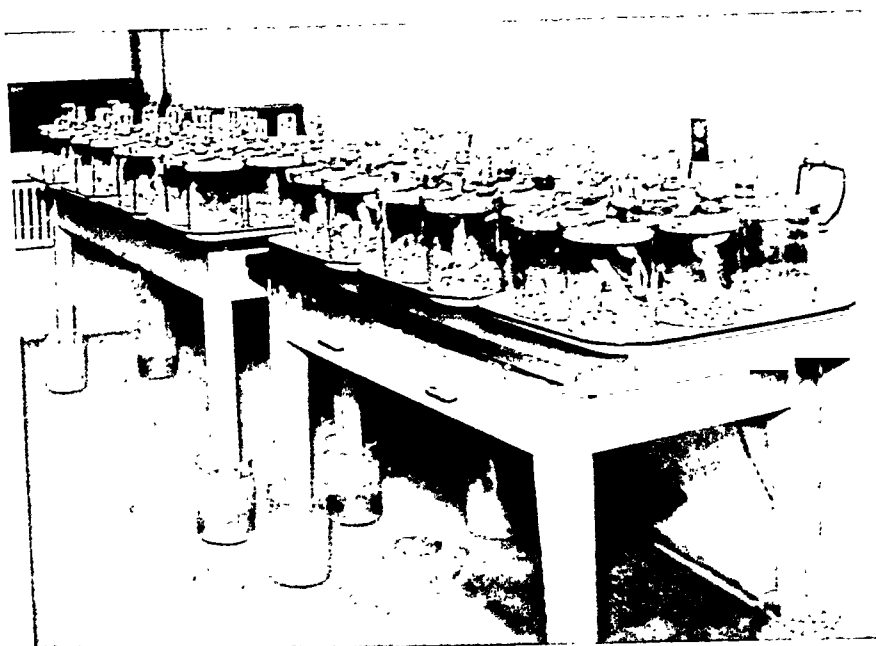


FIG. 1. Photograph illustrating the manner in which mice should be housed when infected with the virus of psittacosis. The tables should not be near a wall and are placed against one in this instance only because a photograph could not have been obtained otherwise.

be observed several weeks, their jars must be cleaned from time to time. Care should be taken not to contaminate oneself during the process of cleansing the jars, and the dirty shavings or bedding in the jars should be sterilized or disposed of in a safe manner.

Criteria for the Presence of Psittacotic Infections in the Inoculated Mice.—The criteria by which the presence of psittacosis in the inoculated mice is determined are

- 1 The development in some or all of the animals of illness which is usually

fatal within 5-14 days, but occasionally not before 30. If after the 4th or 5th day a mouse becomes sick it should be killed rather than allowed to die.

2. The absence of ordinary bacterial infections as determined by aerobic and anaerobic cultures from material obtained at necropsy.

3. The presence in the liver and spleen of the characteristic pathological picture consisting of focal necrotic lesions into and around which there is a collection of polymorphonuclear and mononuclear cells (5).

4. The presence of "minute bodies" of psittacosis (7) in impression smears taken from the liver and spleen—particularly the spleen. These bodies stain easily with our modification of Castaneda's methylene blue safranin method (5).

Phosphate buffer pH 7.0.....	95 cc.
Formalin.....	5 cc.
Loeffler's methylene blue.....	10 cc.

Stain 2 minutes with the methylene blue preparation, rinse in tap water, and quickly counterstain with a 10 per cent aqueous safranin solution. The "minute bodies" take a purple or blue stain while the cells are pink.

5. The establishment of serial passages of the virus in mice by means of liver and spleen emulsions from the animals receiving unfiltered sputum or sputum filtrates.

6. The demonstration that mice which have lived for 30 days following the inoculations of sputum or sputum filtrates have developed an active immunity against a potent strain of psittacosis virus. The tests for immunity should not be made sooner than 30 days after the primary inoculations, because mice develop an immunity against psittacosis slowly.

All of the above conditions obviously need not be fulfilled in each instance, sometimes one, sometimes another serves to establish a diagnosis.

RESULTS

Specimens of sputum from 28 individuals have been examined for the presence of psittacosis virus in the manner described above, and the results of the tests have been summarized in Table I. Most of the specimens of sputum were collected between the 3rd and 9th days of illness. We are reasonably certain that 17 of the 28 persons had psittacosis and that the remaining 11 did not. In the sputum of 12 of the 17 patients with psittacosis active virus was demonstrated; no virus was found in specimens from 4; and the results of the examination of the material from 1 were hard to interpret. No virus was demonstrated in the sputum from the 11 control cases.

A few remarks concerning the negative and doubtful results obtained with sputum from cases of psittacosis will not be amiss. The

TABLE I

Summary of Results of Tests for the Presence of Psittacosis Virus in the Sputum of Human Beings

Number	Name	Psittacosis in patients, summation of evidence	Psittacosis virus, present or absent in suspected birds	Type of human material examined			
				Blood	Sputum	Sputum filtrate	Organ emulsions
1	G. P. B.	+	+				
2	H. B.	+	+	-	+		
3	T. S.	+	+	-	+		
4	M. M.	+	+	-	-		
5	H. K.	+	+	-	+	+	
6	D. K.	+	+		+	+	
7	R. H. L.	+	+		+	+	
8	C. W.	+	+	-		+	
9	M. F.	+	+			+	-
10	M. Su.	+	+	-		-	
11	E. A. S.	Probably	Not tested			+	
12	F. S.	"	Not proved			±	
13	B.	+	" "			-	
14	G. L.	+	Not tested		+	+	
15	A. G.	+	" "		+		
16	Mon.	+	+		+		
17	G. S.	+	Not tested		+		+
			+		-		+

Controls: Patients in whom psittacosis was at first suspected, later events showing definitely that they did not have the disease

1	G. S.	-	-		-	-	
2	E. S.	-	-		-	-	
3	R. C.	-	-		-	-	
4	M. S.	-	-	-	-	-	
5	M. Fa.	-	-		-	-	
6	S. G.	-	-		-	-	
7	B. C.	-	-		-	-	
8	A.	-	-		-	-	
9	L. E.	-	Not tested		-	-	
10	J. W.	-	" "	-	-	-	
11	H.	-	" "		-	-	
			Not proved		-	-	

+ indicates that the patients on summation of evidence had psittacosis, that the virus of psittacosis was found in the birds associated with the human cases, or that the material examined from human sources contained virus.

- indicates that the patients did not have psittacosis, that the virus of psittacosis was not found in the birds associated with the patients, or that the material from human sources did not contain virus.

sputum from Case 8 (C.W.) was collected on the 20th day of illness, 2 days after the patient's temperature had returned to the normal level. In this instance, the fact that the specimen was collected so late in the course of the illness may account for the failure to demonstrate the virus. The specimen from Case 17 (G.S.) sent in for examination was not sputum, but saliva, and this error on the part of the individual collecting the material probably accounts for the negative result, because the patient undoubtedly had psittacosis as virus was later obtained from material secured at autopsy. The 6 mice receiving sputum filtrate from Case 10 (M.Su.) survived, but 4 of them remained perfectly well when tested for immunity by means of injections of potent psittacosis virus. In this test the 6 negative control mice died and the 6 positive control ones survived. We recorded the result as doubtful because we have not had sufficient experience to make a definite laboratory diagnosis of psittacosis on such evidence alone. No obvious reasons were found to account for the failure to demonstrate virus in the specimens of sputum collected from Cases 3 (T.S.) and 11 (E.A.S.).

From 6 of the patients (Table I) with psittacosis, blood was collected and injected (1 cc. in each animal) intraperitoneally into mice. The blood was collected from each case at approximately the same time that the sputum was obtained. In no instance did we recover virus from the blood in spite of the fact that the active agent was demonstrated in the sputum of 5 of the individuals.

Nasal washings (3) from Case 1 (G.P.B.) yielded no virus although it was present in his sputum. Filtered urine and stools from Case 12 (F.S.) were negative, according to tests in mice, for virus, yet it was present in the sputum.

Bits of lungs, liver, and spleen were obtained at the time of autopsy of 3 of the patients. Virus was recovered from material from 2 of them, while in that from the other one none was demonstrated (Table I). The organs that yielded the negative results came from an individual who had been ill 15 days and from whose sputum collected 6 days prior to death virus had been recovered.

The virulence for mice of strains of virus derived from human sputum proved to be fully as great as that of strains derived from

birds. Increasing severity of the psittacotic infection in the rodent host with successive animal passages was manifested in every instance in which serial transfers were made. Bedson and his coworkers (8) and Gordon (9) have stated that the virus derived from human sources has not displayed the same degree of pathogenic stability for mice as that derived from parrots. Because of our divergent experience, we inoculated a variety of strains of mice in order to determine whether a varying susceptibility to psittacotic infection in this host might account for this difference. Moreover, before recommending the mouse as a suitable animal for use in a diagnostic test for psittacosis, it was desirable to ascertain whether different strains of this host vary considerably in susceptibility to the virus. Six strains were, therefore, studied in a comparative way, and all were found to react in essentially the same manner to inoculations of material containing the virus of psittacosis. Furthermore, it was found that very small doses were in general as capable of inducing infection in one strain as in another. Webster's virus-susceptible and virus-resistant mice were not used in this work.

DISCUSSION

From the results reported at this time it is obvious that the sputum collected from patients with psittacosis is an excellent material in which to demonstrate the presence of the etiological agent of the disease. This is accomplished by means of injections of unfiltered washed sputum or sputum filtrates into the peritoneal cavities of mice. We were unable to demonstrate virus in the blood when mice were used as the test animal. The active agent is in the blood, however, and Bedson and his coworkers (1) clearly demonstrated this fact by the use of budgerigars as the test host. Our endeavors, however, have been directed towards the development of a satisfactory diagnostic test in which the mouse, a safe and inexpensive host, can be used instead of birds. It appears that we have been successful, and for more than 3 years no birds have been employed in our laboratory for diagnostic purposes. In addition to ourselves other workers (10) have found the sputum-mouse test satisfactory for the diagnosis of psittacosis in human beings.

SUMMARY

By means of intraperitoneal injections in mice of unfiltered sputum or filtrates of sputum a large percentage of the cases of psittacosis in human beings can be successfully diagnosed in a relatively safe manner.

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A STUDY OF REPEATED ATTACKS OF EXPERIMENTAL PNEUMOCOCCUS LOBAR PNEUMONIA IN DOGS

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PLATES 7 AND 8

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We have described in a previous communication, a method for the production of experimental pneumococcus lobar pneumonia in the dog (1). Briefly this consists in the injection of small quantities of highly virulent pneumococci, suspended in a starch-broth medium, through a radio-opaque catheter inserted into a terminal bronchus with the dog under the fluoroscope. The experimental disease thus produced bore a close analogy, both in its clinical course and pulmonary pathology, to lobar pneumonia seen in the human being. The severity of the disease was, for the most part, found to be dependent upon the amount of culture injected. With doses of less than 0.04 cc. the dogs always recovered. Increasing the dosage above this amount resulted in infections of greater intensity and with a mounting mortality until at 0.25 cc. of culture death usually ensued. With a dose of 0.4 cc. of culture death occurred regularly.

This observed relationship between the infecting dose and the outcome of the experimental disease, within the limits indicated, afforded the possibility of determining with considerable certainty whether a dog which had recovered from one attack of lobar pneumonia was more or less susceptible to a subsequent infection. The present paper embodies the results of such an investigation. We have studied the effects of repeated attacks of lobar pneumonia in twenty-five individual dogs undergoing 78 infections induced at intervals of 3 days to 19 months. The number of attacks to which a single animal was subjected ranged from two to eleven. All the dogs were sacrificed during their final infection for the purposes of studying the pulmonary pathology.

Materials and Methods

The experimental disease was produced with a single type of pneumococcus, namely Type I, Strain A₅, which is a known highly virulent organism. For the initial attack a dose of 0.05 to 0.1 cc. of an 18 hour broth culture was given which produced a moderately severe disease course, lasting 4 or 5 days and clearing up usually by crisis. Doses of 0.02 to 1 cc. of culture were employed in the induction of subsequent infections. These were initiated in the previously involved as well as in the hitherto uninvolved lobes of the lung. One or more X-rays were taken on each dog during the course of the disease. The details of weight, dosage, amount of pulmonary involvement, number of attacks, blood cultures and white blood cell counts are shown in Tables I and II. The dogs killed during their second infection (presented only in Table II) received a final infecting dose of 0.25 cc. with the exception of No. 43E which was given 0.4 cc. of culture.

Survival following Reinfection with Lethal Doses

Thirteen dogs were observed through the course of 33 repeated attacks of experimental lobar pneumonia (Table I). All except one received doses in the lethal range (0.25 to 1 cc. of culture) and all but one survived. This animal, No. 31D, a very old dog receiving 1 cc. of culture, died at the end of 30 hours with a mixed infection consisting of Gram-negative and Gram-positive bacilli as well as pneumococci. That the resistance of the reinoculated animals had been decidedly enhanced as a result of recovery from one attack of pneumonia is shown by a comparison with dogs infected similarly for the first time. Of a series of twenty-two dogs used in this and previous work which received a dose of 0.25 cc. in their primary infection, only five survived and none of four dogs injected with 0.4 cc. lived.¹

Relative Severity of Successive Infections

The recurrent attacks of pneumonia were uniformly mild in character even with a dose of 0.4 cc. or greater. The febrile course was brief, lasting only 2 or 3 days and the lesion was usually confined to a single lobe. As nearly as could be determined, the time intervals elapsing between reinfections, which were 3 days to 19 months, did not alter the relative severity of the reinfections. Dogs 6B and 15B (Table I) received three infections in alternate lobes of the lung within

¹ Since the completion of this study we have infected a number of dogs with a dosage of 0.25 cc. to 0.5 cc. All died except two and these received the largest amount of culture.

a period of 8 days, each infection being given as soon as the temperature reached a normal level after the preceding attack. There were no essential differences between the initial and third attacks.

Occurrence of Bacteremia

One of the most striking differences in reaction between the primarily and secondarily infected animals was that of the occurrence of bacteremia. Daily blood cultures were made on all animals following infection until the time of death or recovery. Of a total of 69 dogs observed in this and other studies (1) during their primary infection, 37 showed invasion of the blood stream by the pneumococcus at some time during their illness, while of a total of 54 recurrent infections, bacteremia occurred in only three instances and was of a minimal nature (Table I). The bacteremia present in the initially infected dogs was frequently marked and in the majority of instances was associated with a fatal outcome often with hundreds of pneumococci per cc. of blood. On the other hand the bacteremia in the recovered dogs amounted at most to only six colonies per cc.

Leucocytic Response

Another difference between the primary and subsequent infections was observed in the leucocytic response. When lethal infecting doses were employed in the initially infected animals there was an early and lasting leucopenia which was usually accompanied by bacteremia. Analogous dosage in the reinfected dogs produced in most instances a prompt leucocytosis (see Table I).

Acquired Pneumococcal-Promoting Activity of the Blood Serum

In order to determine whether acquired humoral immunity played a rôle in the increased antipneumococcal resistance shown by the recovered dogs, observations were made on the pneumococcal-promoting activity of the inactivated serum of six animals following one or more attacks of the disease. The technique for this procedure was the same as that described in earlier studies (2, 3). It was found that acquired immune substances appeared in the serum of five of the six dogs at some time during the period of observation but not after each attack (Table I). In most instances they were not demonstrable

TABLE I

Dog No. and weight	No. of infections	Date of inoculation	Amount of culture injected	Extent of lesion by X-ray	Bacteremia	Outcome of disease	White blood cells	Pneumococidal-promoting activity of dog serum*	
								Serum dilution	Date
6B 7.4 kg.	1	12/ 1/30	0.25	R. L., R. M.	+	R 6 days	11 < 43		
	2	1/26/31	0.02	R. L.	0	R 3 days	8 < 28		
	3	3/ 9/31	0.02	R. U.	0	R 2 days	—		
	4	3/12/31	0.02	L. L.	0	R 2 days	—		
	5	3/16/31	0.02	R. L.	0	R 2 days	—		
	6	4/13/31	0.02	R. L.	0	K 24 hrs.	12 < 20		
10B 9.8 kg.	1	12/29/30	0.25	R. L.	0	R 3 days	15 = 16	1-1280†	12/29/30
	2	7/13/31	0.25	R. L.	0	R 3 days	11 < 19	1-160	1/29/31
	3	12/14/31	0.5	L. L.	0	Not ill	8 = 9	1-160	2/11/31
	4	1/ 6/32	0.25	R. L., R. M., R. U.	+	K 24 hrs.	15 > 6	1-80	12/14/31
15B 14.6 kg.								1-40	12/22/31
	1	1/12/31	0.05	R. U., R. M., R. L.	0	R 5 days	9 < 36	0	1/14/31
	2	3/ 9/31	0.02	R. L.	0	R 3 days	—	0	3/12/31
	3	3/12/31	0.02	L. L.	0	R 3 days	—	0	3/16/31
	4	3/16/31	0.02	R. L.	0	Not ill	—	1-80	3/20/31
	5	6/ 8/31	0.25	R. L.	0	Not ill	12 = 13		
	6	7/ 6/31	0.25	R. L.	0	Not ill	8 = 10	1-20	7/ 6/31
	7	7/27/31	1.0	R. L.	0	R 3 days	11 < 24	1-20	7/21/31
	8	12/ 7/31	0.25	R. M.	0	R 3 days	13 < 34	1-40	12/ 9/31
	9	3/15/31	0.5	R. L.	0	R 3 days	11 < 30	1-40	3/15/31

	10	10/24/32	0.5	L. L.	0	R 3 days	8 < 16	1-20 1-20 1-80 1-40 1-40 1-40	3/16/31 3/17/31 10/24/32 10/25/32 10/26/32 10/28/32
	11	11/ 8/32	0.25	L. L.	0	K 3 hrs.	—		
20R	1	2/ 2/31	0.05	R. L.	0	R 3 days	8 = 10	0	2/ 2/31
11.0 kg.	2	12/14/31	0.35	L. U., L. L.	0	R 4 days	7 < 18	0	2/ 6/31
	3	3/15/32	0.5	L. L.	0	R 2 days	12 < 18	1-320	12/14/31 12/22/31
	4	10/24/32	0.5	—	0	R 3 days	12 < 20	1-40 1-40 1-40 1-40	3/15/32 3/16/32 3/17/32
	5	11/ 7/32	0.25	L. L.	0	K 6 hrs.		1-40 1-20 1-20	10/24/32 10/25/32 10/26/32 10/28/32

R = recovery.

D = died.

< = increase.

> = decrease.

= signifies approximately unchanged.

R.L. = right lower lobe.

R.M. = right middle lobe.

R.U. = right upper lobe.

L.L. = left lower lobe.

L.U. = left upper lobe.

*The figures under the heading "Pneumococidal-promoting activity of dog serum" indicate that dilution of the dog's serum which when added to 0.2 cc. of fresh rabbit serum + rabbit leucocytes is capable of destroying 10^{-6} of the standard suspension of pneumococci (approximately 1000 microorganisms).

† This animal had an injection with pneumococcus autolysate before the first infection.

TABLE I—*Concluded*

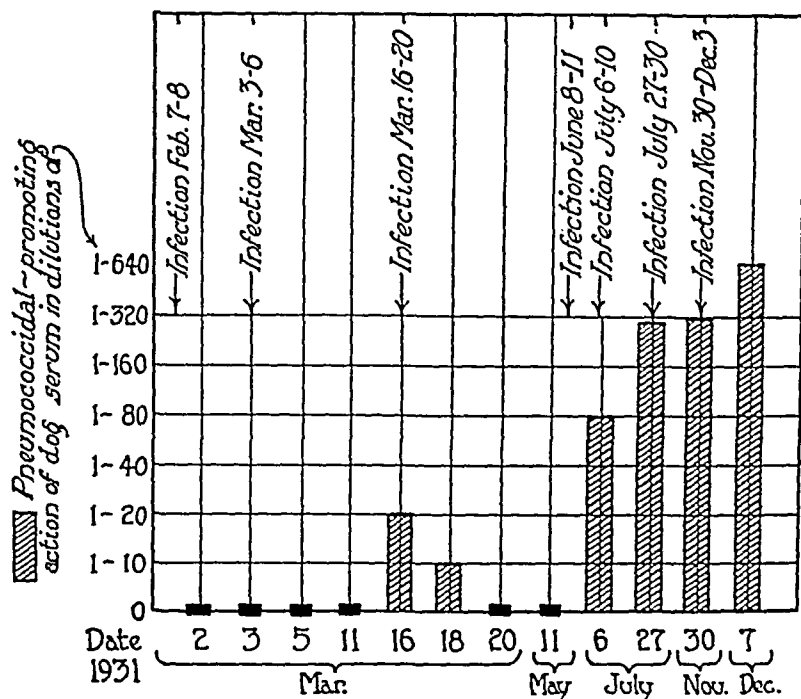
Dog No. and weight	No. of infections	Date of inoculation	Amount of culture injected	Extent of lesion by X-ray	Bacteremia	Outcome of disease	White blood cells	Pneumococcal-promoting activity of dog serum*	
								Serum dilution	Date
22B 7.4 kg.	1	2/ 2/31	0.04	R. L., R. U., R. M., L. U.	0	R 8 days	thousands 8 < 12	0	2/ 2/31
	2	3/ 3/31	0.02	L. L.	0	R 4 days	12 < 22	0	3/ 3/31
	3†	3/16/31	0.25		0	R 3 days	12 < 26	0	3/11/31
								0	3/16/31
								0	3/18/31
								0	3/20/31
23B 7.4 kg.	1	2/ 2/31	0.03	R. L.	0	R 6 days	12 < 18	0	3/ 2/31
	2	3/ 3/31	0.02	L. L., L. U.	0	R 3 days	14 < 26	0	3/ 3/31
								0	3/ 5/31
								0	3/11/31
	3	3/16/31	0.25	R. L.	+	R 4 days	16 < 30	1-20	3/16/31
								1-10	3/18/31
24B 13.6 kg.								0	3/20/31
								0	5/11/31
	4	6/ 8/31	0.25	R. L.	0	R 3 days			7/ 6/31
	5	7/ 6/31	0.25	R. L.	0	R 3 days	13 < 16	1-80	7/27/31
	6	7/27/31	1.0	R. L.	0	R 4 days	14 = 12	1-320	11/30/31
	7	11/30/31	0.25	R. L.	0	R 4 days	12 < 21	1-320	12/ 7/31
	8	1/ 6/32	0.25	R. L.	0	K 12 hrs.	12	1-640	
	1	1/26/31	0.02	R. L., R. M., R. U.	+	R 4 days	15 > 3 < 31		
	2	5/11/31	0.02	R. L.	0	R 4 days	7 < 12		7/ 6/31
	3	7/ 6/31	0.25	R. L.	0	K 24 hrs.	6 = 8	1-20	7/ 7/31

10D 8.9 kg.	1 2 3	11/ 2/31 5/ 2/32 1/20/33	0.05 0.25 0.25	R. L. R. L. R. L.	0 0 0	R 8 days R 4 days K 3 hrs.	8 < 24 9 = 8
31D 8.3 kg.	1 2	1/ 2/32 2/29/32	0.001 1.0	R. L., R. M., R. U. L. U., L. M., R. U.	0 +	R 7 days Died 30½ hrs.	9 < 27 7 > 0.4
5E 11.1 kg.	1 2 3	12/ 7/32 1/ 4/33 1/20/33	0.25 0.35 0.25	L. L. L. L. R. L.	0 0 0	R 4 days R 3 days K 6 hrs.	12 > 8 6 < 18
42E 10.4 kg.	1 2 3 4	12/21/32 1/ 4/33 2/13/33 2/21/33	0.25 0.4 0.4 0.4	L. L. L. L. L. L. R. L.	0 0 0 0	R 5 days Not ill R 3 days K 6 hrs.	7 < 32 16 > 10 14 < 35 8 < 24

† Dog died of injuries a few days later.

§ Dog very old. The lung showed irregular pneumonia due to a mixed infection with Gram-negative and Gram-positive bacilli as well as pneumococci.

until after several reinfections as illustrated in Text-fig. 1 (Dog 23B). With increasing number of recurrences of the disease, immune substances could be detected with greater constancy and tended to persist in the serum for longer periods of time. Repeated tests made during eight attacks of pneumonia in four different dogs (Nos. 15B, 20B, 23B and 24B, Table I and Text-fig. 1) showed that the titer of



TEXT-FIG. 1. The development of pneumococcal-promoting activity of the serum during the period of seven attacks of experimental lobar pneumonia; Dog 23B. "Pneumococcal-promoting action of serum" on day of infection indicates the state of the blood serum just before the induction of the disease.

pneumococcal-promoting substances remained at a constant level or even tended to diminish during the course of a single infection.

It should be pointed out that failure to demonstrate the presence of acquired immune substances in the serum of these dogs at certain times does not exclude their presence since the method is limited in the detection of these substances in low concentrations. In a previous study of acquired humoral immunity in human cases recovering from lobar pneumonia (3), there were several instances in which the in-

activated and diluted serum failed to exhibit pneumococcal-promoting activity at any time while the fresh whole serum showed the development of this property about the time of recovery, suggesting the occurrence of acquired immune substances in low concentration. Other evidence for this inference is presented in the study to which reference has been made. Such parallel tests with the whole fresh dog serum were not considered worth while since the dog's blood normally possesses a relatively high degree of pneumococcal action which tends to persist, often in undiminished degree, throughout the course of the experimental disease. Whether or not our tests failed to reveal the existence of small quantities of acquired immune substances, the results obtained do indicate that the recovered dog's increased resistance to subsequent infection is not dependent on an excess of circulating antipneumococcal immune bodies, because dogs apparently lacking this property of the serum were able to localize the pneumococci just as effectively and recovered from the disease just as quickly as did those exhibiting it to a relatively marked degree.

Acquired immune substances were demonstrable with much greater regularity in the human beings recovering from lobar pneumonia than was the case in dogs but their relationship to the process of recovery is just as obscure.

Pathology

Twenty dogs were sacrificed during their secondary or subsequent attacks for the purpose of studying the evolution of the lesion. A comparison of such lesions with similar aged ones of the primary infection (described elsewhere (4)) revealed certain striking differences in their development.²

3 Hour Lesions.—In two recovered dogs, Nos. 15B and 10D, killed at the end of 3 hours, the lesion differed little, either macroscopically or microscopically, from the process observed at this stage of the primary infection, except that it tended to be slightly more extensive. The involved area was 2 to 3 cm. in diameter, of a diffuse mottled red color and sharply demarcated from the surrounding normal lung tissue. A firm nodule about 1 cm. in diameter was felt in the center of this area. Microscopically there was seen a central area of intra-alveolar

² The stain used was a modification of the Gram-Weigert devised by Mrs. H. M. Wallace and described in our previous study (4).

Pathology of L₁

Dog No.	Age of lesion	Site of lesion in relation to locus of previous infection	No. of previous infections	Interval since preceding infection	White blood cells during final infection	Extent of pulmonary involvement
	<i>hrs.</i>				<i>thousands</i>	
15B	3	Same lobe	9	15 days	—	Lesion 3 cm. diameter
10D	3	Same lobe	2	8½ mos.	—	Lesion 2-3 cm. diameter
20B	6	Same lobe	4	11 days	—	L.L. ¼ consolidated
42E	6	Same lobe	3	5 days	8 < 24	R.L. ¾ consolidated
43E	6	Same lobe	1	3 wks.	6 < 8	R.L. ⅔ consolidated. R.M. and R.U. small areas consolidated
4C	6	Same lobe	1	3 wks.	16 < 20	R.L. ¼ consolidated
5E	6	New lobe	2	13 days	10 (at death)	R.L. ¾ consolidated
46E	14	Same lobe	1	3 mos.	10 > 5	R.L. ⅔ consolidated
6C	15	Same lobe	1	4 wks.	16 < 27	R.L. ⅓ consolidated
27H	16	Same lobe	1	3 wks.	? < 13	R.L. small area 3 cm. diameter
38D	12	New lobe	1	8½ mos.	—	L.L. almost completely consolidated
15H	15	New lobe	1	7½ wks.	16 > 7	L.L. completely consolidated. L.U. beginning consolidation
6B	24	Same lobe	5	26 days	12 < 20	R.L. completely consolidated
10B	24	Same lobe	3	3 wks.	15 = 14	R.L. { completely R.M. { consolidated R.U. partly consolidated

Killed during Final Infection

Histological study of lesions				Relationship between macrophages and pneumococci
Intensity of cellular infiltration	Degree of macrophage reaction	Distribution of macrophages	Presence of pneumococci	
re like 6 hr. primary lesion than 3 hr.	None	None	+	None
out as 3 hr. lesion	None	None	+	None
ense in center. Resembles 12-15 hr. primary lesion	Beginning	Perivascular and septal; generalized	+	None
ense. Resembles 12-15 hr. primary lesion	Well marked	Perivascular and septal; generalized	0	Macrophage reaction marked; pneumococci absent
ense. Resembles 12-15 hr. primary lesion	Well marked	Perivascular and septal; generalized	+	Where macrophage reaction well marked no pneumococci seen
y moderately intense but more like primary lesion at 12-15 hr. than 6 hr.	None	None	+	None
t intense. Resembles 6 hr. primary lesion	None	None	+	None
ense	Moderate	Perivascular and septal; generalized	+	Where macrophage reaction well established, pneumococci few or absent
ense	Well marked	Perivascular and septal; generalized	0	Macrophage reaction marked; pneumococci absent
ense	Beginning	Perivascular and septal; generalized	0	None
t intense	None	None	+	None
ense	None	None	+	None
derately intense	Moderate	Perivascular and septal; chiefly near pleura	0	Macrophage reaction marked; pneumococci absent
ense	Moderate	Perivascular and septal. Beginning general	+	? if pneumococci fewer where macrophage reaction well marked

Dog No.	Age of lesion	Site of lesion in relation to locus of previous infection	No. of previous infections	Interval since preceding infection	White blood cells during final infection	Extent of pulmonary involvement
	<i>hrs.</i>				<i>thousands</i>	
24B	24	Same lobe	2	7 wks.	6 < 8	R.L. $\frac{1}{3}$ to $\frac{1}{2}$ consolidated
3E	24	Same lobe	1	4 $\frac{1}{2}$ mos.	10 > 0.5	L.L. { completely L.U. { consolidated R.L. { beginning R.M. { consolidation
11G	24	Same lobe	1	5 wks.	11 > 3	R.L. completely consolidated
16G	24	Same lobe	1	2 $\frac{1}{2}$ mos.	12 > 5	R.L. completely consolidated R.M. $\frac{3}{4}$ consolidated. R.U. small area consolidated
22H	24	New lobe	1	5 wks.	13 < 29	R.L. $\frac{2}{3}$ consolidated
4H	24	New lobe	1	8 wks.	11 > 2	L.L. almost completely consolidated. R.U. $\frac{1}{2}$ consolidated

cluded

Histological study of lesions				Relationship between macrophages and pneumococci
Intensity of cellular infiltration	Degree of macrophage reaction	Distribution of macrophages	Presence of pneumococci	
None	Moderate	Septal chiefly. Irregularly distributed	0	Macrophage reaction present; pneumococci absent
None but very hemorrhagic	Beginning	Septal, chiefly near pleura. Little perivascular reaction	+	Where macrophage reaction well marked, pneumococci few or absent
None	None	None	+	None
Very moderately intense	None	None	+	None
Here irregular	Beginning	Perivascular and septal	+	Where macrophage well established, pneumococci absent
Very moderately intense	None	None	+	None

Dog No.	Age of lesion	Site of lesion in relation to locus of previous infection	No. of previous infections	Interval since preceding infection	White blood cells during final infection	Extent of pulmonary involvement
	<i>hrs.</i>				<i>thousands</i>	
24B	24	Same lobe	2	7 wks.	6 < 8	R.L. $\frac{1}{3}$ to $\frac{1}{2}$ consolidated
3E	24	Same lobe	1	4 $\frac{1}{2}$ mos.	10 > 0.5	L.L. { completely L.U. { consolidated R.L. { beginning R.M. { consolidation
11G	24	Same lobe	1	5 wks.	11 > 3	R.L. completely consolidated
16G	24	Same lobe	1	2 $\frac{1}{2}$ mos.	12 > 5	R.L. completely consolidated. R.M. $\frac{3}{4}$ consolidated. R.U. small area consolidated
22H	24	New lobe	1	5 wks.	13 < 29	R.L. $\frac{2}{3}$ consolidated
4H	24	New lobe	1	8 wks.	11 > 2	L.L. almost completely consolidated. R.U. $\frac{1}{2}$ consolidated

included

Histological study of lesions				Relationship between macrophages and pneumococci
Intensity of cellular infiltration	Degree of macrophage reaction	Distribution of macrophages	Presence of pneumococci	
Intense	Moderate	Septal chiefly. Irregularly distributed	0	Macrophage reaction present; pneumococci absent
Intense but very hemorrhagic	Beginning	Septal, chiefly near pleura. Little perivascular reaction	+	Where macrophage reaction well marked, pneumococci few or absent
Intense	None	None	+	None
Very moderately intense	None	None	+	None
Border irregular	Beginning	Perivascular and septal	+	Where macrophage well established, pneumococci absent
Very moderately intense	None	None	+	None

cellular infiltration surrounded by a zone of edema-filled air sacs. The cellular exudate was composed of polymorphonuclear leucocytes and red blood cells. In one dog, No. 10D, pneumococci were abundant in all portions of the lesion, lying chiefly within the alveoli, but also present in the alveolar walls. On the other hand, very few pneumococci could be detected in the pulmonary process in Dog 15 and these were all intracellular. In this respect it differs from the primary lesion at 3 hours.

6 Hour Lesions.—By the end of 6 hours the character of the inflammatory reaction in the lungs of the recovered dogs began to show certain distinct differences from similar aged lesions of the initial infection. In the gross, the lesions were larger and of a firmer consistency. In several instances they comprised three-fourths to four-fifths of the entire lobe (Table II), whereas primary lesions of this age, and produced by equivalent dosage, involved less than half the lobe. On microscopic examination, the four dogs reinfected in a previously involved lobe showed an intensity of intra-alveolar cellular infiltration analogous to that observed in the 12 to 15 hour stage of the primary process. Likewise there was less generalized hemorrhage and more perivascular and peribronchial edema than seen at the end of the 6 hour period in the first infection.

The most striking histological change at this time was the appearance of numbers of large mononuclear cells present in the alveolar septa, the perivascular tissues and free in the intra-alveolar exudate. This cellular reaction, exhibited by three of the four dogs, was similar to that which we have described previously (4) as occurring wherever resolution of the pneumonic process is taking place. In the primary infection it has not been observed to occur before 48 to 72 hours after the initiation of the experimental disease except in very occasional instances where small areas of beginning resolution have been detected at 24 hours. The several stages in the development of this characteristic response of the fixed tissue cells are shown in Figs. 1 to 4. The first manifestations of the reaction are an increase in the number and size of large mononuclear cells in the septa and an accumulation of mononuclear cells around the smaller blood vessels (Figs. 1 and 2). Certain of these cells are seen to be protruding into the alveolar spaces. As the process develops, the number of large cells in the alveolar walls increases, producing a definite thickening of these structures (Fig. 3). There is a progressive liberation of the large mononuclears into the air spaces where they are observed to assume the functions of the free histiocyte or macrophage. With the further evolution of the reaction these cells replace the polymorphonuclears

and become the predominant cell of the thinning pneumonic exudate (Fig. 4). While we have no evidence that all or even the greater part of the newly appearing large mononuclear cells in the tissues are potential macrophages, we do know from the cytological studies which Kredel and Van Sant made in this laboratory³ that the majority of the large mononuclears in the exudate of the resolving pneumonic process are true macrophages. The term "macrophage reaction" which we have employed to designate this phenomenon includes both the structural and functional changes observed.

The lesion in two of the three dogs showing the macrophage reaction was produced within the limits of time observed to be necessary for the lung to regain its normal structure following recovery; *i.e.*, 1 to 2 weeks. Thus the cellular picture in Dog 42E, reinfected 5 days after recovery from the preceding attack, represents to a large extent a subsiding macrophage reaction as evident by the characteristic changes seen in the uninvolved tissue. However, Dog 20B reinoculated 11 days subsequent to the antecedent infection showed in all probability a newly developing fixed tissue cell reaction since the lung tissue around the inflamed area appeared normal.

The fifth "6 hour" dog (No. 5E) which received its second infection in a lobe not previously involved, showed a pulmonary lesion essentially similar to the 6 hour primary process.

Pneumococci were found in the lesions of four of the five dogs killed at this time and were most abundant in that of the animal infected in a previously uninvolved lobe. Phagocytosis, by both polymorphonuclear leucocytes and macrophages within the alveolar exudate was observed. Heart's blood cultures made at the time of autopsy were sterile in every case.

12 Hour Lesions.—Of the five dogs sacrificed at the end of 12 to 16 hours, three, Nos. 46E, 6C and 27H, were infected in a previously involved lobe while the other two, Nos. 38D and 15H (Table II) received their infecting dose in a hitherto uninvolved lobe. The resulting inflammatory reactions differed both macroscopically and microscopically in the two sets of animals. The lesions produced in lobes which had been the site of a previous process were smaller and more intensely consolidated than were those occupying lung tissue involved for the first time. They had apparently progressed little in extent from the 6 hour stage but had increased decidedly in density. On microscopical examination the intensity and uniformity of the intra-alveolar cellular exudate was considerably greater in the former group of dogs. Likewise these three dogs showed a generalized macrophage

³ These authors, in a study to be published soon, made differential counts of the vitally stained cells in the inflammatory exudate of dogs killed at various stages of experimental lobar pneumonia.

reaction which was not observed in the lesions of the other two. In two of the three lesions occupying the site of an earlier infection, pneumococci could not be found, either in microscopic sections or by culture. This early sterilization of the inflammatory process is in marked contrast to the abundant and widespread presence of pneumococci in the corresponding primary lesion produced by the same or smaller amounts of culture. The lesion of the third dog of this group showed about as many microorganisms as were observed in the newly involved lobes. The pneumococci were few or absent in those areas where cellular infiltration was intense and abundant at the growing edge of the lesion. Heart's blood cultures made at autopsy were sterile in every case.

24 Hour Lesions.—Eight dogs were sacrificed at the end of 24 hours. Of this number six received their infection in a lobe previously involved while in two the final lesion was produced in an area which had not been the site of earlier infection. The clear-cut differences in the character of the inflammatory reaction of these two kinds of tissue loci that were observed in the 6 and 12–16 hour lesions could not be detected at this stage of the process. The extent of involvement in dogs reinfected in the same lobe exceeded in three instances that in the two animals infected in a new lobe. In all three of the former dogs there was extension of the process to other lobes while in only one of the two latter dogs did this occur. Bacteremia occurred in one case in each group. The dosage was the same in all these instances. Microscopic examination likewise failed to reveal any consistent difference in the lesions produced at these two sites. The character of the lesion in three of the dogs, Nos. 11G, 16G and 4H, differed in no essential way from the primary process of the same age produced by doses of culture from which recovery regularly ensues; *i.e.*, the alveoli were moderately distended with an exudate consisting principally of polymorphonuclear leucocytes with a varying number of red blood cells, while here and there were air sacs partly or completely filled with edema fluid. Five of the dogs, Nos. 3E, 6B, 10B, 24B and 22H, however, showed a macrophage reaction in varying degree. In four this cellular change was widespread, a condition which has not been observed in the primary process at 24 hours.

In the lesions of two of the dogs showing a macrophage reaction, pneumococci were not found, either by microscopic examination or culture. In the other two with a similar degree of this type of cellular response, pneumococci were present in the lesions but not abundant. On the other hand, pneumococci in considerable numbers were observed to be distributed throughout the inflammatory area in the three dogs showing no macrophage reaction. In the lesion of Dog 3E, pneumococci were very few or absent in the oldest part which showed an early beginning of the macrophage response but were present in large numbers in the more recently involved areas characterized by a cellular reaction predominantly polymorphonuclear in nature. An analogous distribution of pneumococci in relation to the presence of macrophages was observed in the pulmonary lesion of Dog 22H.

The differences observed in pathogenesis between the primary infection and the secondary or subsequent ones may be summarized as

follows: Secondary lesions initiated in the lobe previously affected evolve much more rapidly than do the primary ones during the first 12 to 16 hours. They are characterized by the early appearance of a generalized macrophage reaction and a marked diminution in numbers of pneumococci in the tissues or their complete disappearance. On the other hand, if the recurrent infection has been initiated in a lobe not involved in the first attack the histological picture of the lesion is like that of the primary infection produced with a non-fatal dose. However, it differs markedly from a primary infection caused by an equivalent dose, in that the cellular infiltration is much more intense and even, there is less hemorrhage, the pneumococci are fewer in number and a greater proportion are intracellular. By the end of 24 hours there was little further change in the lesions except spreads to other lobes which occurred in some instances. The dogs sacrificed at this time showed more diversity in their pulmonary pathology than did those studied at an earlier stage. Some of the lesions resembled those of a similar age in the primary infection but the majority showed the cellular picture and the distribution of pneumococci described above which is seen only in the lung at the time of recovery and has not been observed in the initial process until the end of 48 to 72 hours.

The number of instances in which spread to other lobes occurred in this series of eight animals sacrificed at 24 hours is much higher than that observed in the animals allowed to survive. It is true that X-ray is not as informative as actual inspection but it will reveal any extent of consolidation. It is of interest in this connection to note that more than half this series of dogs showed a drop of white count instead of the customary rise observed in the great majority of reinfected dogs. Dog 3E, showing the most marked involvement, had the greatest degree of leucopenia. Yet the blood of this animal was sterile at autopsy. A marked leucopenia during a primary infection has, in our experience, always been accompanied by bacteremia. What the outcome of the disease in Dog 3E would have been we cannot certainly say since with the single exception noted earlier none of the reinfected dogs have died, even with doses much larger than this animal received. Dogs infected for the first time have not survived when more than half the total lung area was involved.

DISCUSSION

Prior to the recent important contribution of Finland and Winkler (5) our knowledge concerning immunity following recovery from lobar pneumonia was very meager. These authors have reported the detailed study of 57 cases undergoing recurrent attacks of the disease. As a result of this we now have for the first time concise information concerning the susceptibility of human beings to repeated infection with pneumococci of the same type. Their data show that the distribution of pneumococcus types in the recurrent attacks of lobar pneumonia is about the same as that occurring in the primary infection; *i.e.*, one attack of the disease does not confer on the individual permanently increased resistance against a subsequent pulmonary infection with the homologous type pneumococcus. Nor on the contrary does it render them more susceptible. The recurrent attacks with all types were of about the same intensity and duration as the initial ones, although bilateral involvement and atypical or bronchopneumonias were more common and the incidence of bacteremia slightly less in the former.

Our findings in dogs appear at first sight to differ from these observations in human beings. However, analysis of the two sets of data brings out the fact that they cannot be compared closely since the time intervals between attacks are not analogous. In Finland and Winkler's series of twenty Type I pneumonias undergoing twenty-one recurrent attacks caused by the same type pneumococcus, only one occurred in less than a year after the first attack while in our experimental series all but one of the 54 repeated infections were produced at intervals of less than a year—most of them a few weeks to several months apart. May not the infrequency of secondary attacks of lobar pneumonia noted by Finland and Winkler during the first year or so following the preceding infection have been due to a temporarily increased antipneumococcal resistance?

The only available experimental data which bear directly on this subject are those of Cecil and Blake (6). These authors, in a study of active immunity following experimental pneumococcus pneumonia in monkeys, found definite differences between Type I on the one hand and Type III and Group IV on the other. Reinfection of two

monkeys recovered several weeks previously from a Type I pneumonia resulted, in both instances, in a very mild transient disease course, while several animals recovered from pneumonia due to Type III and Group IV developed just as severe and prolonged a pneumonia following a second infecting dose of the homologous pneumococcus as they had shown in the first attack.

Our experiments show clearly that one attack of lobar pneumonia confers on the dog considerably increased power to localize and limit the spread of a second pulmonary infection produced by the same type pneumococcus. To what altered reactivity on the part of the dog's tissues is this heightened resistance to be attributed? The inconstant presence of demonstrable humoral immune substances in the blood of dogs recovered from one or more attacks of the experimental disease makes it difficult to ascribe the dogs' increased immunity to this factor alone. Again, the occurrence of leucocytosis accompanying the secondary attacks following infecting doses which at the the initial attack regularly produced a leucopenia, undoubtedly indicates a favorable response on the part of the diseased animal. But it seems unlikely that this reaction could have been the determining element in the control of the disease process since the increase in the number of circulating leucocytes was no greater than observed often in the initial infections and occasionally it was only slight.

The most significant and possibly the only definite evidence of the nature of the recovered dog's increased resistance lies, we believe, in the characteristic changes in the fixed tissue cells observed in the pulmonary lesion early in the course of the secondary or subsequent infections. The basis for associating this reaction with heightened tissue immunity to the pneumococcus rests on the following observations: (1) The transformation of the septal and perivascular histiocytes, or macrophage reaction, occurs regularly at the time of recovery from the initial attack of experimental lobar pneumonia and is accompanied by resolution. Whether this reaction is of primary or secondary importance in the process of recovery we do not know, but the fact that it begins in the secondary lesion while the inflammatory process is still extending, and that this secondary process is of more limited extent and of briefer duration than the primary one suggests that it plays a rôle in the control of the infection. (2) Our observa-

tions on the distribution of pneumococci in the lesions of the rein-fected dogs showed consistently that microorganisms were diminished in number or absent in the regions where the macrophage reaction was most pronounced, and not infrequently lesions showing this reaction to a well developed degree were found to be sterile on culture. Primary lesions of a similar or much greater age were always found to contain pneumococci—usually in considerable numbers. (3) The finding that the macrophage response occurs earlier in the lesions produced at the site of previous infection than it does in secondary lesions initiated elsewhere in the lung indicates at least an increased reactivity of the local tissue cells to the pneumococcus. As to whether the action of the macrophages is principally phagocytic in nature or whether they exert other effects we have no data.

It must be granted that our information on this subject is incomplete. Certain animals of the series, particularly some among those killed at 24 hours after the inception of the infection, failed to reveal the characteristic reaction of the fixed tissue cells yet showed by the degree of localization of the lesion that they possessed increased resistance to the pneumococcus. We suspect from our observations that there are marked individual variations in the time at which this reaction occurs as well as in its degree. Time elapsing between infections may well play a rôle in the histological response although the time factor, within the limits observed, did not appear to be important in the degree of resistance exhibited by the dogs to reinfection.

The observations of other workers on the macrophage response of lungs to the presence of pneumococci are of much interest in connection with this work. Stuppy, Cannon and Falk (7) found that rabbits immunized by means of the intrabronchial insufflation of killed pneumococci, Types I and II, survived lethal doses of the homologous microorganism similarly administered. Examination of the lungs of such rabbits killed within 24 to 48 hours after the infecting dose showed a marked proliferative and exudative type of reaction in which the macrophage was the predominant cell of the inflammation. They inferred from their data that this local cellular response of the lung was definitely associated with increased specific immunity to the pneumococcus. In a study of local immunization of the lungs to the staphylococcus and *B. coli*, Tuttle and Cannon (8) found a

marked proliferation of the macrophages and a similar distribution in the form of perivascular collars that we observed in the lungs of our dogs undergoing recurrent pneumonia. Fried, who has made extensive investigations on the macrophage system of the lungs (9), particularly in relation to the tubercle bacillus, stresses the importance of the rôle played by the macrophages in the lungs' defense against pathogenic microorganisms.

SUMMARY

A study has been made of repeated attacks of experimental lobar pneumonia in the dog produced by the intrabronchial injection of *Pneumococcus* Type I. Twenty-five individual dogs were given 78 infections in all at intervals of 3 days to 19 months. The number of attacks to which a single animal was subjected varied from two to eleven. It was found that recovery from this experimental disease conferred on the animal increased resistance against subsequent infections as shown by the fact that such animals regularly survived doses of culture which in the dog infected for the first time produced a fatal outcome. The recurrent attacks of pneumonia were uniformly mild in character; the febrile course was brief; the pulmonary lesion was usually confined to a single lobe, and bacteremia seldom occurred. There was no detectable difference between the second and the subsequent infections, which could be produced whenever desired, nor did the time intervals between attacks appear to bear any relationship to the severity of the experimental disease. Tests for acquired anti-pneumococcal immune substances in the blood after recovery showed their presence in some animals and not in others, yet dogs without demonstrable humoral immunity appeared to be just as resistant to reinfection as those possessing it.

A comparison of the pathogenesis of these secondarily induced lesions with those of the initial infection revealed certain striking differences between the two. Secondary lesions produced in the lobe previously affected tended to evolve much more rapidly than did the primary ones. They were characterized by the early appearance of a generalized macrophage reaction and a marked diminution in the numbers of pneumococci in the tissues or their complete absence. These changes occurred more slowly in secondary lesions initiated in

hitherto uninvolved lobes. The macrophage reaction, which consists of a swelling of the fixed tissue cells (histiocytes) and a subsequent liberation of macrophages into the alveolar exudate, is regarded as a significant evidence of increased antipneumococcal resistance, since it has been observed to occur regularly at the time of recovery from the first infection and is accompanied by the local disappearance of the invading microorganisms.

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EXPLANATION OF PLATES

The stain used was a modification of the Gram-Weigert devised by Mrs. H. M. Wallace.

PLATE 7

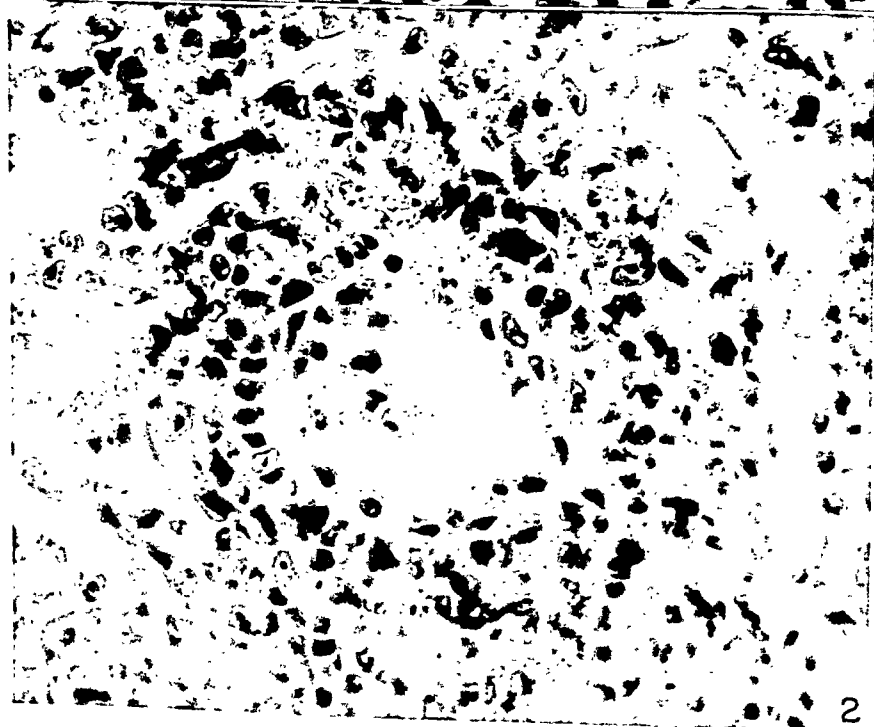
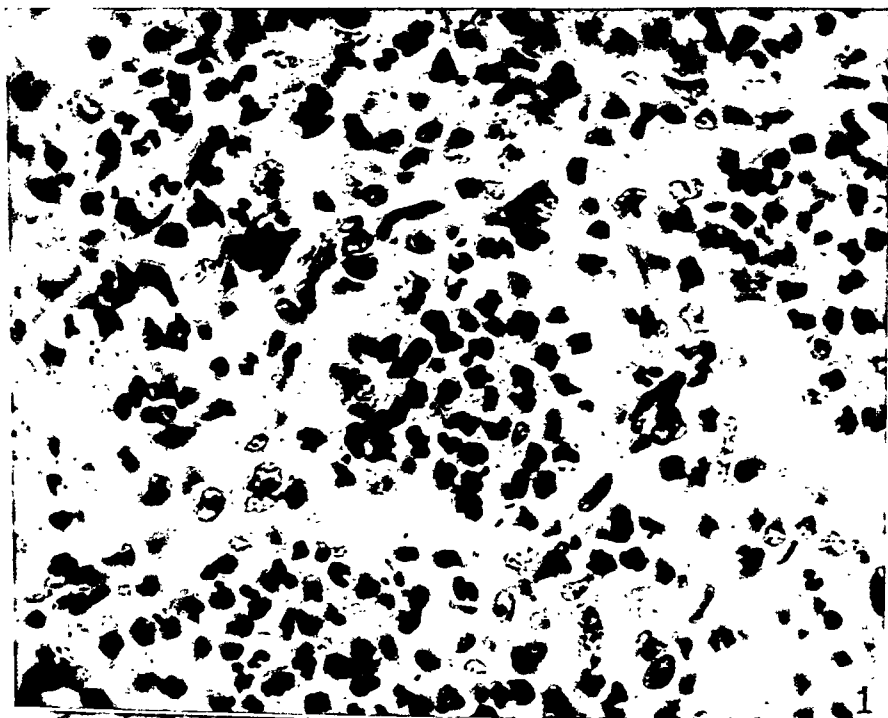
FIG. 1. Dog 27H. Killed 16 hours after the inception of the experimental disease. Lesion shows beginning macrophage reaction as indicated by an increase in the number and size of the large mononuclear cells in the alveolar walls. $\times 750$.

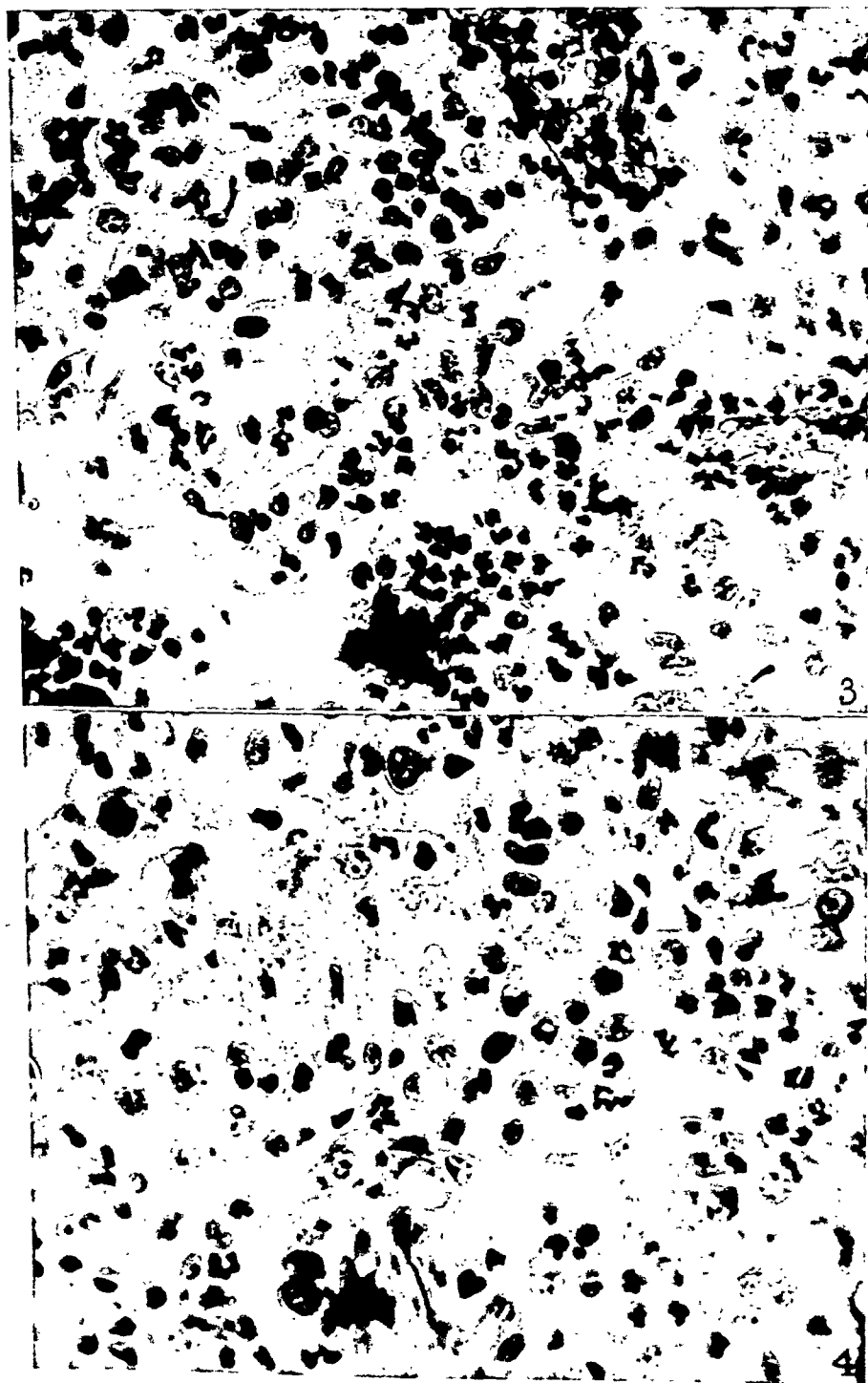
FIG. 2. Dog 27H. Accumulation of mononuclear cells around a small blood vessel; another manifestation of a beginning macrophage reaction. $\times 600$.

PLATE 8

FIG. 3. Dog 43E. Killed 6 hours after the inception of the experimental disease. Lesion shows thickening of the alveolar walls characteristic of the evolving macrophage reaction and due principally to the presence of large mononuclear cells. $\times 640$.

FIG. 4. Dog 43E. Area of well advanced macrophage reaction. Many large mononuclears have been liberated into the alveoli where they assume the appearance of macrophages. $\times 650$.





(Coombs and Roberts: Repeated attacks of experimental pneumonitis)

TESTS FOR PNEUMOCOCCUS HYPERSENSITIVENESS IN DOGS AFTER RECOVERY FROM EXPERIMENTAL PNEUMOCOCCUS LOBAR PNEUMONIA

By LOWELL T. COGGESHALL, M.D.

(From the Department of Medicine of The University of Chicago, Chicago)

(Received for publication, November 19, 1934)

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Lauche (1) has pointed out that infants under 4 months of age seldom contract lobar pneumonia. This apparent insusceptibility he attributes to their not yet having had opportunity to become sensitized to the pneumococcus. He contends that as individuals grow older they come into contact with the pneumococcus or even contract mild unrecognizable infections until an occasion may occur when the microorganisms invade the lung tissue and produce a marked infection. The basis of this infection in a sensitized individual is the localization of pneumococci in the hilum lymph nodes or hilum, producing an inflammation which obstructs the lymph flow and reverses its direction toward the periphery thereby carrying the organisms into the lung parenchyma. Lausche believes that the rapid onset of the pneumonic infection is an allergic phenomenon occurring in a sensitized individual.

Wadsworth (2) was the first to note that after intratracheal inoculation with pneumococcus cultures a localized inflammation was produced only in animals previously immunized by means of killed cultures of pneumococci. Recently Sharp and Blake (3) have shown that pneumococcus autolysates produce an exudative inflammatory pulmonary lesion in sensitized rabbits, and that there is a close parallelism between cutaneous and pulmonary hypersensitiveness.

From the observations of Tillett and Francis (4) it was shown that a protein-free, type-specific polysaccharide of the homologous pneumococcus elicits an immediate wheal type of reaction when injected into the skin of patients recovering from pneumococcus lobar pneumonia. However, Finland and Sutliff (5) obtained skin reactions with the specific pneumococcus polysaccharides in a number of hospital patients who had no recent history of pneumonia.

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these animals has been described in the preceding study of which this series of dogs formed a part (13).

At various intervals, from 2 days to 2 months following recovery from the initial or recurrent attacks, Type I pneumococcus autolysate was injected directly into the previously affected lobe by the use of the radio-opaque catheter. The initial dose was 0.5 cc. Later 1.0 cc. was administered. The autolysate was also injected intracutaneously in amounts of 0.2 cc. to determine the presence or absence of skin sensitivity. Temperature and pulse rates, white blood cell counts, total and differential, X-rays of the lungs and reinfection were utilized to detect increased sensitivity. Tests for the pneumococcal-promoting activity of inactivated serum were also carried out with a view to determining the possible relationship of acquired immune bodies to allergy. The method used for determining the presence of this serum property was that devised by Robertson and Sia (14). Mixtures of heated diluted dog serum and washed rabbit leucocytes and serum seeded with varying numbers of pneumococci were agitated in the incubator and tested for sterility at the end of 72 hours.

Preparation of Autolysates.—The autolysates were prepared according to the method used by Sharp and Blake (3). The preparation was standardized on the basis of nitrogen content. In the various lots the content of nitrogen ranged from 134 to 160 mg. per 100 cc. of autolysate. The potency of the autolysate was ascertained by testing rabbits sensitized to the pneumococcus. Intracutaneous injection of these animals was followed in 24 hours by the characteristic urticarial reaction.

EXPERIMENTAL

Pulmonary Reactions.—Seven dogs received intrapulmonary injections of the pneumococcus autolysate at varying time intervals of 1 week to 2 months following an attack of the experimental pneumonia. Four of the seven dogs, Nos. 6B, 15B, 22B, and 23B, showed a definite shadow by X-ray at the site of the injection. The three remaining dogs, Nos. 2B, 3B and 10B, which had received similar previous infections, showed no pulmonary lesion by X-ray. The pulmonary lesions when present were best seen 24 hours after injection. At the end of 48 hours they were clearing up or had disappeared entirely. The X-ray shadows averaged 4 cm. in diameter and in no single instance were they large enough to conform to the boundaries of any one lobe. There was no difference in the size of the lesion whether the dogs were injected within 1 week after an infection or if they were allowed to rest as long as 2 months. Also, dogs with repeated autolysate injections reacted in the same manner on each occasion. Likewise, there was

to the pneumococcus by the above described method was not attended by cutaneous allergy to derivatives of the pneumococcus used for immunization. At the same time Mackenzie and Woo (7) injected guinea pigs with an alkaline pneumococcus extract. In about two-thirds of their animals an allergy was produced similar to the allergic response of the tubercle bacillus, but the animals showed no significant alteration in susceptibility to pneumococcus infection by intraperitoneal inoculation. Zinsser and Mallory (8) proved that guinea pigs can be artificially sensitized to bacterial products. Furthermore, they believe that an actual infection is a better means of sensitizing an animal than is the artificial administration of bacterial antigens. Bull and McKee (9) definitely showed that rabbits having recovered from an acute pneumococcus infection were hypersensitive to an autolysate of the homologous organism. This hypersensitive state was observed within 48 hours and lasted as long as 4 months. Also rabbits immunized with killed and living organisms were found to be hypersensitive to the pneumococcus autolysate. However, immunization by the above means did not produce as high a degree of sensitivity as did infection. Reimann (10) has recently published an excellent critique of the literature relating to allergy and pneumococcus lobar pneumonia.

We have made an attempt to throw further light on this subject by observing the pulmonary and cutaneous reactions to pneumococcus autolysate in dogs recovering from experimental pneumococcus lobar pneumonia. As far as we can find there have been no studies of pneumococcus hypersensitiveness in this animal. The experimental disease produced in the dog, which we have described elsewhere (11), resembles human lobar pneumonia more closely than that so far induced in any other animal with the exception of the monkey (12).

Methods and Materials

Male dogs were used exclusively. Their weights ranged from 10 to 15 kilos. They were healthy in external appearance and very active. All were isolated for a preliminary period of at least 1 week and temperatures taken; any animal with an elevated temperature was discarded. They were fed a normal diet of meat, carrots and bread ground into a hash, plus cod liver oil, the latter given three times weekly.

Fourteen dogs, as shown in Table I, were infected with Type I and Type II virulent pneumococci. Dosages ranging from 0.02 to 1 cc. of culture suspended in a broth-starch mixture were placed directly into a terminal bronchiole of the lung by means of a No. 11 F. ureteral radio-opaque catheter with the dog under the fluoroscope (11). The animals were infected on twenty-three different occasions; three of them, Nos. 6B, 15B and 23B, received four or more infections each, at various time intervals. The character of the initial and subsequent attacks in

these animals has been described in the preceding study of which this series of dogs formed a part (13).

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[illegible]

3/16/31	Inf.	I	0.02	I.T.	I.D.
+	present.	0 = absent.	- = not done.	I.T. = intratracheal.	I.D. = intradermal.

- * Acquired immune substances in terms of pneumococcus + rabbit dilution of the heated dog serum which when added to 0.2 cc. of fresh rabbit serum + rabbit destroying 10⁻⁶ of the standard suspension of pneumococci (approximately 1000 microorganisms).

TABLE I—*Concluded*

Dog No.	Date	Infection or auto-lysis	Type of pneumo-coccus	Amount of culture injected	Route of inoculation	Leucocytosis	Elevation of temperature	Increase in pulse rate	Skin reaction	Titer of acquired immune substance*	Definite X-ray lesion	Additional data
22B	2/ 2/31	Inf.	I	cc. 0.04	I. T.	+	+	+	—	0	+	Recovered 8th day. Very ill. Entire left side involved
	2/24/31	Aut.	I	1.0 0.2	I. T. I. D.	+	0	+	0	0	+	Lesion in R.L. lobe 2 cm. diameter at 24 hrs.
	3/ 3/31	Inf.	I	0.02	I. T.	+	+	+	—	0	+	Moderately ill. Recovered 4th day. R.L. lobe consolidated
	3/16/31	Inf.	I	0.25 1.0	I. T. I. T.	+	+	+	—	0	+	Recovered 3rd day. R.L. lobe consolidated
	4/ 7/31	Aut.	I	0.2	I. D.	0	0	+	0	0	+	Lesion in R.L. lobe 3 cm. diameter
23B	2/ 2/31	Inf.	I	0.02	I. T.	+	+	+	—	0	+	Recovered 4th day. Mild infection R.L. lobe
	2/24/31	Aut.	I	1.0 0.2	I. T. I. D.	+	0	+	0	0	+	Lesion R.L. lobe 3 cm. diameter
	3/ 3/31	Inf.	I	0.02	I. T.	+	+	+		—	+	Recovered 3rd day. R.L. lobe
	3/16/31	Inf.	I	0.25	I. T.	+	+	+		—	+	Recovered 4th day. R.L. lobe
	7/27/31	Inf.	I	1.0	I. T.	+	0	+		—	+	Recovered 3rd day. R.L. lobe
36B	4/ 7/31	Aut.	I	1.0 0.2	I. T. I. D.	0	0	0	0	—	+	Control dog
37B	4/ 7/31	Aut.	I	1.0 0.2	I. T. I. D.	0	0	0	0	—	+	Control dog

25D	3/ 2/32	Aut.	I	1.0 0.2	I. T. I. D.	0	0	0	0	-	+	Last infection 6 months previously. Killed after 24 hrs.
34D	3/ 2/32	Aut.	I	1.0 0.2	I. T. I. D.	+	0	0	0	-	+	Recovered 10 days previously. Killed after 24 hrs.
42D	3/ 2/32	Aut.	I	1.0 0.2	I. T. I. D.	0	0	0	0	-	+	Given Type I antipneumococcus serum (20,000 units). Killed after 24 hrs.
3N	3/ 2/32	Aut.	I	1.0 0.2	I. T. I. D.	0	0	0	0	-	+	Normal dog. Killed after 24 hrs.

no relationship between the severity of the preceding infection and the presence or extent of the autolysate lesion in the lung.

In the control series, three normal dogs, Nos. 36B, 37B and 3X, showed a definite pulmonary lesion by X-ray after autolysate injection. The appearance of these lesions was indistinguishable from those seen in the recovered dogs. In two other normal dogs, Nos. 9B and 10B, no reaction to the autolysate could be detected.

Cutaneous Response to the Autolysate.—Observations on the skin tests were recorded every 2 hours for the 1st day and then twice daily for 1 week after intradermal injections of the autolysate. A positive response was not elicited in any of the animals. In one dog (No. 15B) there was a small hemorrhagic area at the site of injection at 12 hours which, however, had none of the characteristics or appearance of a wheal reaction.

Reinfection.—It has been shown in this series of dogs and in the preceding report (13) that the second and subsequent attacks of experimentally induced lobar pneumonia are not only no more severe but are definitely milder than the first infection. Two dogs, Nos. 22B and 23B, were infected on three different occasions, each inoculation being done as soon as the previous infection had cleared up. On the last inoculation the dose was 0.25 cc. of culture, one that was usually lethal. However, each infection was very mild and the course of the disease after the large dosage was also mild, terminating by crisis on the 4th day. An inoculation of 1 cc. of a culture, known to be lethal in this amount under ordinary circumstances, was then given to Dog 23B, which likewise promptly recovered on the 4th day.

Two dogs, Nos. 9B and 10B, were given an injection of autolysate before the first inoculation. All of the remaining animals were inoculated previous to autolysate administration. Subsequent infections in both groups were similar in intensity and in duration. There was no evidence to suggest that administration of the pneumococcus autolysate had altered the disease in any manner.

Acquired Humoral Immunity.—In five of the animals repeated tests of the pneumococcal-promoting activity of the dogs' serum were made before and after infection and intrabronchial autolysate injection. Two of the five dogs tested (Table I) showed the presence of acquired humoral immune substances when the autolysate was given.

In both these animals immune substances appeared in the blood after the initial autolysate injection, which was made before the first infection. Subsequent infections did not increase the titer of these immune bodies. In the third dog, No. 15B, acquired humoral immunity did not appear until after the third attack of the experimental disease. Thus the presence or absence of humoral immune substances could in no way be associated with the lack of detectable hypersensitivity to the pneumococcus.

Temperature, Pulse, White Blood Counts.—No febrile reaction was observed in any of the animals after intratracheal or intradermal administration of autolysate, but in many instances the pulse rate increased for a short period of time. Leucocytosis as noted in the protocol was usually present. However, it has been shown in a later experiment that normal dogs after an intramuscular injection of morphine develop an elevated white count. The explanation of this is probably that most of the morphinized dogs salivate, vomit and have several loose stools, thereby becoming dehydrated. Eosinophilia was not observed in any of the animals.

Pathology.—Four dogs were given intrapulmonary injections of 1 cc. of pneumococcus autolysate and were killed at the end of 24 hours for the purpose of microscopical examination (Table I). The time interval of 24 hours was selected because fluoroscopy revealed that the pulmonary lesion was of the greatest extent at that time. In this experiment the same lot of Type I autolysate was used in all four animals. Dog 3X was presumably a normal dog; No. 25D was a dog that had recovered from a Type I pneumococcus pneumonia 6 months previously; No. 34D had recovered from a similar infection 10 days before; and Dog 42D was given 25 cc. of unconcentrated Type I antipneumococcus serum 6 hours before administration of the autolysate.

The lesions in all four dogs were of a deep mottled red color with irregular edges. They were approximately 3 cm. in diameter and on palpation slightly crepitant.

On microscopical examination certain differences between the lesions of the two recovered dogs on the one hand, and the normal and immune serum-treated dogs on the other, were noted. The inflammatory exudate, of rather a diffuse nature in all four animals, was

definitely more cellular in the two dogs recovered from previous infection and resembled a lobular pneumonia, partly coalescent. In the areas of intense cellular infiltration polymorphonuclear leucocytes predominated. In the less infiltrated parts a considerable number of mononuclear cells were present—many of the macrophage type. Some of these were observed to be arising locally but there was no definite thickening of the alveolar walls such as is observed regularly when macrophages appear in the exudate at the time of recovery from experimental lobar pneumonia (15). However, the lesions in both these animals showed marked perivascular accumulations of large mononuclear cells, characteristic of the macrophage reaction, which we have described as occurring early in the evolution of the secondarily induced (recurrent) infections (13).

The exudative process in the normal dog and the dog previously injected with type-specific antipneumococcus serum was more edematous and hemorrhagic than that above described. Large mononuclear cells were fairly frequent in the cellular exudate but a smaller percentage of these appeared to be typical macrophages. While the lung of the normal dog showed slight, if any, increase in the number of perivascular mononuclear cells, this reaction was present in the lesion of the serum-treated animal although not nearly as marked as that observed in the recovered dogs.

An adequate estimate of the significance of these differences would require considerably more data than we have obtained. However, the results of this and previous studies suggest that the fixed cells of the lung tissue which have been subjected to a previous pneumococcus infection, react much more quickly to the presence of the pneumococcus or its products than do the cells of the normal lung.

SUMMARY AND CONCLUSIONS

The data derived from the above described investigations indicate that dogs do not develop hypersensitivity to the pneumococcus as the result of experimental lobar pneumonia. This inference is based on the following findings:

1. Fifteen dogs were given Type I and Type II pneumococcus lobar pneumonia and following recovery were tested for hypersensi-

tiveness by means of intrabronchial and intracutaneous injections of the autolysate made from the homologous pneumococcus.

2. Seven dogs showed a pulmonary lesion discernible with the X-ray at site of the autolysate inoculation; three of these dogs were normal controls.

3. No evidence of a positive skin reaction was found in any of the fifteen dogs, many of which received repeated infections and intradermal autolysate injections.

4. Subsequent infections in the same animals were definitely milder than the initial infection.

5. The infections following the administration of intrapulmonary and cutaneous autolysate were practically of the same intensity as the initial infection.

6. Temperature, pulse rates, white blood counts and differential blood pictures showed no significant variations following intrapulmonary injection of autolysate.

7. Tests for the acquisition of humoral immune bodies following autolysate injection and recovery from the experimental disease showed the presence of these substances in some of the dogs and their absence in others.

8. Study of the pathology of the pulmonary lesions produced by the autolysate failed to reveal histological changes characteristic of an allergic reaction. However, the presence of perivascular accumulations of large mononuclear cells observed in the lesions of the recovered dogs does suggest a locally accelerated reactivity of the fixed tissue cells to the products of the pneumococcus.

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THE DEVELOPMENT OF PURE CULTURES OF FIBROBLASTS FROM SINGLE MONONUCLEAR CELLS

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PLATES 9 TO 11

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Fibroblastic or fibroblast-like transformation of blood mononuclear leucocytes in tissue culture was first reported by Awrorow and Timofejewskij (1). Carrel and Ebeling (2), working with chick buffy coat explants, believed the transformation to be an adaptive phenomenon of the monocyte to an unfavorable environment, since the fibroblast is a more resistant cell. Fischer (3) found that special conditions, such as adding dead muscle tissue to the cultures, were necessary for fibroblastic metamorphosis of chick mononuclear cells. Maximow (4), in a detailed study of fibroblastic growth from guinea pig, monkey, rabbit and chick buffy coat explants, observed that there were certain species differences and that guinea pig non-granular leucocytes transformed into fibroblasts most readily. Bloom (5) also described polyblastic and fibroblastic mutations of rabbit lymph cells in tissue culture.

While studying the reaction of cells from infected animals to bacterial products (6), it was again observed (Figs. 1 and 2) that luxuriant growths of fibroblasts frequently developed from guinea pig buffy coat explants. Beginning fibroblastic growth usually occurred 4 to 6 days after explantation. Further analysis indicated that there was a definite correlation between the age of the animal, cellular type and fibroblastic growth potentialities of the explants. Buffy coats from younger animals contained a higher percentage of mononuclear cells, particularly lymphocytes, and also developed greater fibroblastic growths.

The object of the present study was to determine whether pure cultures of fibroblasts derived from single cells could be developed by

using mononuclear cells as progenitors. This seemed feasible because fibroblastic transformations occurred readily, and suitable suspensions of individual mononuclear cells could be made with minimal trauma.

Unsuccessful efforts, chiefly by Fischer (7), to grow pure cultures of fibroblasts from single cells were ascribed by him to unsatisfactory environmental conditions or to manipulative trauma of the cells. Burrows (8) stated that while single cells may move in plasma medium they do not grow, and expressed the opinion that cells can grow only when crowded together in an environment where accumulation of certain normal metabolic products acts as a stimulus for proliferation.

EXPERIMENTAL

Preparation of Mononuclear Cellular Suspension.—Several methods for obtaining a mononuclear cellular suspension were tried and discarded before a satisfactory plan was perfected. Cells aspirated into a fine capillary pipette from minced buffy coat failed to grow, probably because of the excessive trauma entailed in the manipulation. A mononuclear exudate consisting almost entirely of monocytes and clasmotocytes was then produced by the injection of paraffin oil intrapleurally into medium sized guinea pigs. The exudate, removed aseptically by opening the thorax of the pig 5 days after the oil injection, contained many mononuclear cells filled with ingested oil droplets; and although some of them apparently proliferated in tissue cultures this method was discarded for one yielding more normal exudative cells.

The best mononuclear cell incitant for our purposes was the intrapleural injection of low melting point wax. Normal guinea pigs weighing between 300 and 400 gm. were lightly etherized. A small incision was made through the clipped skin of the right chest on the lateral aspect in the mid-thoracic region. Using a medium caliber needle 0.5 cc. of fluid parowax (a widely used commercial wax for household purposes, melting point 48–50°C.) was injected into the pleural cavity. At body temperature this wax congeals into a soft pliable mass. Caution was taken not to inject the wax while too warm. The injection was performed quickly and with minimal trauma, since, as indicated later, the avoidance of even slight hemorrhage into the pleural cavity was necessary for satisfactory experimental conditions. After 3 to 14 days the mononuclear exudate was removed; the most satisfactory time was found to be 5 to 7 days, because after more extended periods various amounts of amorphous debris accumulated, probably due to degenerated cells, and this seemed to affect proliferation of isolated cells adversely. The animal was killed by a sharp blow on the head and exsanguinated as completely as possible by severing the neck vessels. After the heart had ceased beating the thorax was opened under aseptic precautions and great care was taken to prevent red blood cells from entering the pleural cavity. No macroscopic

exudate was visible about the pliable mass of wax. The right pleural cavity was then gently irrigated with 1 or 2 cc. of Tyrode's solution and the suspension removed. A properly prepared cellular suspension was practically water-clear and contained few gross particles. A cell count of the suspension was then made and serial dilutions in Tyrode's solution were so arranged that 0.1 cc. of the suspension contained from one hundred to several thousand cells.

Supravital studies of the cells with neutral red and Janus green, according to the method described by Sabin (9), showed them to be almost entirely mononuclears chiefly of the monocytic type with a smaller percentage of lymphocytes and a few clasmatoctyes. The proportion of lymphocytes ranged between 5 to 20 per cent, the higher percentage resulting from more prolonged pleural irritation. The monocytes were of the normal resting type with large neutral red rosettes and presented no other noteworthy characteristics. Most of the cells were free, but they occasionally adhered to one another in clumps. Rarely, desquamated serosal cells were found in groups or small sheets and they presented the usual polygonal shape with small oval or round nuclei. Polymorphonuclear leucocytes were seldom observed except when the cellular suspension was blood-tinged.

Culture Media.—Plasma was obtained from normal medium sized guinea pigs by heparinizing and centrifuging chilled cardiac blood. 0.5 cc. of a 1 to 700 dilution of heparin in Ringer's solution was used for each 4 cc. of blood. The separated plasma was again centrifuged at high speed to remove erythrocytes. Plasmas showing even slight hemolysis were not used.

The tissue extract was derived from the spleens of the normal guinea pigs that had been previously bled by cardiac puncture. The spleens were freed of adherent fat, finely minced with scissors and suspended in Tyrode's solution to make a 10 per cent tissue extract. The suspension was agitated by aspirating with a pipette and allowed to stand for $\frac{1}{2}$ hour, then centrifuged at high speed for 10 minutes to throw down the gross particles. The supernatant slightly turbid fluid which still contained many blood platelets was further centrifuged for 20 minutes at high speed to remove the thrombocytes, and then appeared quite clear and free from cells. In some experiments the splenic extract was also repeatedly and rapidly frozen in a salt-ice mixture and thawed to obviate the theoretical objection that isolated living cells may still have been present in the extract after prolonged centrifugation.

Carrel's micro flasks were found most suitable for photographic purposes. After repeated trials the following amounts of media and cells in each flask were determined to be most favorable: 0.9 cc. of heparinized plasma, 0.1 cc. of suspension of mononuclear exudative cells in Tyrode's solution and 0.5 cc. of 10 per cent splenic extract. Each flask was shaken to mix the contents thoroughly, then stoppered, following which coagulation soon occurred. The number of mononuclear cells in different flasks varied from one hundred to several thousand. Cultures were set up and placed in an incubator regulated for 37°C. within a short time after removal of the cells from the animal.

The isolated cells, designated for microscopic study, were identified under low power magnification by dots of India ink or black enamel paint applied with a fine pen at suitable locations on the outer surface of the flask. The cultures were placed in a warm chamber during microscopic study and except when being photographed were protected from strong light with a green filter.

Cultures observed for a week or more were washed with Tyrode's solution and renourished with 0.5 cc. of 10 per cent guinea pig splenic extract twice a week. For subcultures, colonies of cells were excised in the usual manner and transplanted into Carrel micro flasks containing 1.0 cc. of 50 per cent guinea pig plasma in Tyrode's solution and 0.5 cc. of 10 per cent guinea pig splenic extract.

A Leitz "micca" photomicrographic attachment for an ordinary microscope was used for recording the changes observed in the cultures. The magnifications obtained by different combinations of lenses were 25, 40, 90 and 200, the last being secured with a water immersion lens.

RESULTS

Types of Cellular Development.—Under suitable experimental conditions some of the isolated mononuclear cells divided by mitosis and proliferated to form colonies of morphologically typical fibroblasts. The development of different cells in tissue culture seemed to be conditioned to a certain extent at the time of explantation, for within a few hours after the cultures were made, distinct differences could be noted in many of the isolated cells: some were dark and very granular with distorted nuclear and cellular outline; obviously they were dying or dead, for they soon fragmented and disintegrated completely. By far the greater proportion of the cells hypertrophied, extended pseudopodia and undulating membranes, and became typical migrating macrophages (Fig. 22). These cells varied greatly in size, rapidly changed their shapes and usually migrated actively. After several days some of them became less active, retracted their pseudopodia, assumed round or irregular polygonal shapes, became filled with dark coarse granules or droplets and eventually degenerated. Others of the macrophage type maintained their healthful appearance and activity for much longer periods. The occasional small clump or sheet of desquamated serosal cells showed no signs of activity and did not proliferate. Admixed red blood cells usually disintegrated within 2 days after explantation.

A smaller percentage of the cells slowly developed into spindle or stellate forms with a varying number of long, pointed, branching,

protoplasmic processes. The nuclei were usually oval or round and contained several nucleoli. These cells did not migrate but altered their shape by rearrangement of their processes. Many of them were marked for identification, photographed and observed for evidences of cellular division. Cells thus selected, however, only occasionally underwent division, but usually degenerated.

Selection of Isolated Cells.—A much better technique with greater chances for success was to search for isolated single cells in the process of mitosis (Figs. 3 to 7), since we were then assured of some proliferation, if only of slight extent. It was discovered that the most favorable period for finding these cells in mitosis was 24 to 48 hours after explantation; although occasionally some isolated cells did not undergo mitosis until after a latent period of 3 days. Diligent and frequent observations of the flasks on the 1st and 2nd day after explantation usually revealed several satisfactorily isolated cells in mitosis in each flask. Such cells were readily recognizable from their characteristic appearance. The cell drew in its processes and assumed a nearly spherical shape; the cytoplasm condensed into large irregular dark masses surrounding a nucleus which had lost its ordinary structure of the resting state. Under a water immersion lens the breaking up of the spireme thread into chromosomes and migration of the chromosomes were frequently observed.

Rate of Proliferation.—The rate of proliferation of individual cells varied even in the same flask. In some instances after proliferation had set in, the number of cells was roughly doubled in each 24 hours. Some proliferated more slowly and others more rapidly. One isolated cell was the progenitor of seven cells in the course of 28 hours; but this rapid proliferation was not maintained. No instances of amitotic division were observed.

Criteria of Fibroblasts.—Cells presenting the following morphological characteristics were considered to be of the fibroblastic type: the cells had spindle, stellate or polygonal shapes with variable numbers of pointed or branch-like protoplasmic processes and relatively large, clear, oval nuclei containing one or several nucleoli. These cells tended to grow in solid sheets or in reticular arrangement and displayed marked cytotropism, but did not have migratory ameboid movements.

Types of Colony Formation.—The type of colony produced from various individual cells differed. Some formed solid sheets of cells (Fig. 18); others developed typical branching formation (Fig. 23). Likewise the morphology of individual cells in different colonies was variable, but different individuals of the same colony were essentially the same. Some fibroblasts had many long branching protoplasmic processes (Fig. 21); others were polygonal with few processes and grew in tight solid sheets (Fig. 24). The oval or round nuclei contained two to six large nucleoli. Probably the different types of cell colony formation can be explained in part by the supporting structure on which the cells grew; proliferation on the free surface of the clot, in the fibrin network or between the glass and coagulum would naturally have their respective influences.

Transitional cells between the typical macrophage and the stellate and spindle-shaped fibroblast were frequently encountered. Some were polygonal with nuclei similar to that of fibroblasts. Variable amounts of granules were present in the cytoplasm. Usually there was a broad, clear, peripheral zone free of granules. Pointed protoplasmic processes were very few and usually entirely absent. Such cells grew in colony formation as solid sheets (Fig. 24).

Although the fibroblasts did not migrate in the sense applied to macrophages, still in an actively proliferating colony, movements and changes in the relationship of cells to one another were often quite marked. This was especially noticeable in colonies growing in branch-like arrangement. From hour to hour the pattern changed, assuming various bizarre shapes. A cell would break one of its connections with a neighbor and later attach itself to another cell. Due to the marked cytotropism, however, individual cells of actively growing colonies derived from single cells seldom severed connections with the mother colony. There was also a marked attraction of one colony of fibroblasts for another, as shown in Figs. 15 to 18. Tongues of cells sent out from each colony soon resulted in complete fusion.

The extent of proliferation of individual cells was variable even in the same flask where the environmental conditions and isolation of the cells were essentially the same. Sometimes macroscopic colonies consisting of hundreds of cells developed from a single cell, while a neighboring cell produced a colony of only ten to twenty members

which then degenerated (Fig. 27). Other cells disintegrated after one or two divisions.

Relationship between Bulk of Medium, Number of Explanted Cells and Cellular Proliferation.—The number and size of the colonies of fibroblasts originating in the flasks containing the larger number of explanted cells was often disproportionately greater when compared with other flasks containing serial dilutions of the same cellular suspension. This suggested that there was an optimal relationship between the bulk of the medium, the number of explanted cells and the degree of cellular proliferation. For this reason, and also because the proportion of mononuclear cells which eventually developed into colonies of fibroblasts was comparatively small, it hardly seemed feasible to isolate single cells from the suspension with a capillary pipette, transfer them to culture media and obtain growth.

The occasional small clump of explanted cells proliferated rapidly. Larger clumps behaved in some respects similarly to explanted buffy coats. Shortly after explantation numerous macrophages wandered out, and fibroblastic forms appeared within 48 hours (Fig. 26). This was much more rapid than that noted in guinea pig buffy coat explants, from which the first spikes of fibroblastic growth usually did not appear until 4 to 6 days after explantation. Proliferating cells that were not well isolated but were in the vicinity of other fibroblasts showed the usual cytotropism, readily united with their neighbors and soon formed large groups.

Subcultures of Fibroblasts.—Colonies of fibroblasts originating from mononuclear cells were easily transplanted, carried through repeated subcultures and still maintained their morphological fibroblastic characteristics (Fig. 25). Attempts have not been made to carry these transplants on indefinitely; and efforts to transform fibroblasts back to macrophages (10, 11) have not been pursued.

Proliferation of isolated macrophages was not observed in these experiments; but persistent efforts in this direction were not made because the migratory character of the cells rendered detection of these changes difficult with our technique. Once a typical migrating macrophage with a broad undulating membrane approached a colony of proliferating spindle-shaped fibroblasts and became entangled in the network of cells. After 48 hours this cell lost its undulating membrane

and underwent division; it subsequently produced a small nest of transitional type of cells similar to those shown in Fig. 24, amongst the fibroblasts. Many other macrophages in close proximity to colonies of fibroblasts did not transform.

Because of the difference in the behavior of the various mononuclear cells an attempt was made to correlate their reaction to vital dyes with their subsequent behavior in culture media. This did not meet with success, as neutral red and Janus green, even in dilutions of 1 to 40,000, proved lethal for these cells.

Inhibiting Influence of Erythrocytes on Cellular Proliferation.—Early in this work when the results of individual experiments were not usually successful, analysis of the variables was undertaken with the object of removing the inhibiting factor or factors. It soon appeared that when there were many red blood cells in the mononuclear cellular suspension the growths were characterized by very slight cellular proliferation; in other words, the explanted mononuclear cells seemed definitely inhibited by the presence of erythrocytes. Disintegration of red blood cells, which usually occurred within 48 hours after explantation, was followed by degenerative changes of the mononuclear cells. The macrophages seemed more affected by the toxic products and usually disintegrated before the fibroblastic forms. Some of the larger macrophages remained viable for longer periods. The transformed fibroblasts also became very dark, developed vacuoles and coarse granulations and their processes became attenuated; proliferation was much inhibited and usually entirely suppressed, and in a few days the cells eventually fragmented.

Several experiments were undertaken to analyze the apparent inhibition of disintegrated erythrocytes on cultures of isolated mononuclear cells. Normal heparinized whole guinea pig blood in a final dilution of 1 to 2,000 had a definite inhibitory effect on growth of mononuclear cells, as described in the preceding paragraph. On the other hand, the soluble products of a 1 to 2,000 dilution of hemolyzed, stroma-free, guinea pig blood produced no inhibition but possibly stimulated growth slightly. The direct effect of erythrocytic stroma alone could not be adequately tested because of the difficulty encountered in resuspending it. The results indicate, however, that the toxic or inhibiting factor of disintegrating red blood cells resides in the stroma.

With improved technique, particularly by avoiding traumatic hemorrhage in the pleural cavity when injecting the wax, satisfactory results were usually obtained.

DISCUSSION

The transformation of guinea pig non-granular leucocytes and mononuclear exudative cells into fibroblasts was again demonstrated. The experimental conditions most favorable for this transformation seemed to be the same as those optimal for the cultivation of other guinea pig fibroblasts. Special conditions such as those described by Carrel and Ebeling (2) and Fischer (3) in their studies of fibroblastic transformation from chicken buffy coats were not necessary in the case of guinea pig cells.

From a study of the development of individual isolated mononuclear exudative cells in tissue culture it is suggested that the type of development or transformation of each cell is more or less conditioned at the time of explantation, because different cells, in apparently the same environment, developed differently, and usually maintained their characteristics. Under the conditions of these experiments most of the cells behaved as typical macrophages. A smaller proportion took on fibroblastic characteristics which persisted. Whether these two divergent cellular morphological groups originate from two different types of cells, such as the monocyte and the lymphocyte, or whether they represent merely different developmental stages of a common cell type was not ascertained in these experiments. The latter conjecture seems most probable in view of the fact that cells in various transitional stages between the macrophage and the typical fibroblast were frequently seen.

It is known that optimal environmental conditions vary in the case of different types of cells. Plasma is the optimal medium for macrophages, while high concentrations of tissue juice are toxic for them. Fibroblasts, on the other hand, thrive in media containing relatively large amounts of embryonic or tissue extract. Parker (11) has observed the transformation of a long established strain of chick fibroblasts into typical macrophages by changing the nutritional environment from one containing embryonic tissue juice to a pure plasmatic medium. He, therefore, considers that the fibroblast and the macrophage represent extreme functional and structural variations of the

same cell type. It thus seems probable that the culture medium used in our experiments, due to the relatively large amount of tissue extract, was more favorable for prolonged fibroblastic proliferation than for continued growth of macrophages. Yet it should again be emphasized that fibroblastic transformation took place soon after explantation when most of the macrophages appeared to be in good condition. This suggests that the different individual cells were variously conditioned at the time of explantation.

This study clearly shows that certain single isolated mononuclear cells of the guinea pig can give rise to a pure colony of fibroblasts. Direct apposition of other cells is not necessary, at least in the case of guinea pig mononuclear cells, to supply the stimulus for cellular division as suggested by Burrows (8) and Fischer (7). It seemed possible that sufficient growth-promoting substance was present in the splenic extract to initiate mitosis in isolated cells. On the other hand, it should be emphasized that a distinct period of latency followed explantation of isolated cells before which mitosis did not occur; and cellular division usually did not begin until a lapse of 1 or 2 days, and sometimes not until several days after explantation; this indicates that the cell may have been elaborating some product into its immediate environment which made it favorable for cellular proliferation. It is also possible that comparatively far removed cells secreted substances into the medium which by diffusion reached the isolated cell and stimulated it. This possibility is suggested by the disproportionately greater proliferation in flasks containing the larger number of mononuclear cells. A definite optimal relationship between the bulk of medium, number of cells and amount of proliferation has also been shown in the case of yeast (12) and Infusoria (13). Wildiers (12) designated this cell-stimulating material elaborated by normal yeast as "*la substance enigmatique*." Robertson (13), working with Infusoria, described an "X substance" produced by the organisms which catalyzed their reproductive rate. He also pointed out the protective effect of mutual contiguity of cells. It seems possible in the light of our experiments that a similar agent is necessary for cellular proliferation of isolated mammalian cells. Likewise it is well recognized that a condition of crowding of the cells in tissue culture is the more favorable one for proliferation, and Burrows' experiments

(8) showed that the continual removal of certain substances produced by normal cells inhibited proliferation.

A distinct cytotropism between cells of the fibroblastic type was noted, and although these cells do not possess true ameboid movement, the growth of small colonies was directed toward similar cells in the vicinity. Proliferation was definitely enhanced by this union. Rous and Jones (14) and others have also pointed out the cytotropism of fibroblasts in tissue culture. This behavior is in distinct contrast to that of the macrophages which usually migrate away from one another.

The question arises as to whether isolated animal cells from other sites of origin might have the property of proliferation in tissue culture or whether this ability is peculiar to the cells selected in this study. Possibly the wax stimulated certain cells to take on fibroblastic transformation potentialities and enhanced their proliferation. Explanted clumps of mononuclear exudative cells gave rise to fibroblastic growth much earlier than did buffy coat explants, suggesting either a stimulation of the exudative cells by the wax or an inhibition of the fibroblastic precursory cells in the buffy coat by the many red blood cells incorporated in the explants. An important contributing factor to the success of our experiments was the minimal trauma associated with the handling of pleural exudative cells. This was relatively slight as compared with techniques used in isolating single cells from other tissues.

Rather exacting conditions were found necessary to accomplish the development of pure colonies of fibroblasts from single cells, and these were in the direction of optimal conditions for guinea pig fibroblasts in general. Aside from inflicting minimal trauma on the cells, the removal of inhibitory factors from the tissue culture media was obviously necessary. The inhibitory effect of erythrocytes or their disintegration products on cellular proliferation was shown and superficially studied. Our observations are in accord with Earle's (15) experiments with the effect of light on blood and tissue cells. He showed that erythrocytes, leucocytes and fibroblasts degenerated when exposed to irradiation, and noted that the disintegration products of red blood cells increased the degenerative changes in fibroblasts. If an amount of irradiation which had practically no effect on fibro-

blasts in the absence of erythrocytes was applied to similar cultures of fibroblasts in the presence of red blood cells, marked degenerative changes took place. Earle also believed that the disintegration products of red cells possibly caused degenerative changes in leucocytes. Our results corroborated this opinion. Further analysis indicates that the toxic factor in disintegrating erythrocytes resides in the lipoid-containing stroma.

Our studies show that when environmental conditions are favorable, and when manipulative trauma is reduced to a minimum, certain isolated cells will proliferate to form pure colonies of fibroblasts. Repeated subcultures of these pure colonies of fibroblasts may be carried on. It is also suggested that with better knowledge of the requisite environmental factors together with suitable methods for isolating other tissue cells, pure strains derived from single cells of various tissues may possibly be developed.

SUMMARY

1. Most isolated guinea pig mononuclear exudative cells in tissue culture become typical migrating macrophages, but a small proportion take on fibroblastic characteristics, and produce pure colonies of fibroblasts. These fibroblasts maintain their morphological characteristics through repeated subcultures.

2. It is suggested that the subsequent development of individual mononuclear cells in tissue culture is conditioned at the time of explantation.

3. Apposition with other cells is not necessary for the initiation of mitotic cellular division.

4. There is a definite optimal relationship between the bulk of the medium, the number of explanted cells and the extent of proliferation. The presence of other cells in the vicinity enhances cellular division.

5. Mitosis in the isolated explanted cell is preceded by a latent period. The rate of division varies in different colonies of fibroblasts.

6. Admixed erythrocytes in the mononuclear suspension definitely inhibit proliferation of fibroblasts in tissue culture. The inhibiting factor in disintegrating erythrocytes is apparently present in the stroma.

JOHANNES K. MOEN

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EXPLANATION OF PLATES

PLATE 9

- FIG. 1. Fibroblastic growth from guinea pig buffy coat 6 days after explantation. Wandering cells are also present. $\times 120$.
- FIG. 2. Type of fibroblastic growth 48 hours after transplantation of culture shown in Fig. 1. $\times 90$.

PLATE 10

- Figs. 3 to 14 inclusive. These photomicrographs show only the cells that have arisen from the single isolated cell in Fig. 3.
- FIG. 3. Photomicrograph showing degree of isolation of a mononuclear cell in mitosis (anaphase) 2 days after explantation, 10.45 a.m. $\times 180$.
- Figs. 4 to 7 inclusive. Higher magnifications showing further stages in mitotic division. FIG. 4, taken at 10.45 a.m.; FIG. 5, at 10.53 a.m.; FIG. 6, at 11.05 a.m.; and FIG. 7, at 11.30 a.m. $\times 400$. The indistinctness of cellular outline in FIG. 5 is due to the marked activity associated with the "boiling" stage.
- FIG. 8. 9 hours after Fig. 7. Two cells in resting stage. 8.15 p.m. $\times 180$.
- FIG. 9. 3 days after explantation. Three cells, 10.30 a.m. $\times 180$.
- FIG. 10. 4 days after explantation. Six cells, 11.30 a.m. $\times 180$.

FIG. 11. 5 days after explantation. Fourteen cells, 2.30 p.m. $\times 180$.

FIG. 12. 6 days after explantation. Twenty cells, 10.30 a.m. $\times 90$.

FIG. 13. 7 days after explantation. About thirty-five cells. $\times 90$.

FIG. 14. 8 days after explantation. $\times 90$.

PLATE 11

FIGS. 15 to 18 inclusive. Lower magnifications of the same colony shown in preceding photomicrographs. Portions of two other colonies of fibroblasts are seen sending out tongues of cells and uniting with the first colony. Several wandering macrophages are visible. Fig. 15, 9 days; Fig. 16, 10 days; Fig. 17, 12 days; and Fig. 18, 13 days after explantation. $\times 40$.

FIGS. 19 and 20. Still lower magnification of combined colonies of fibroblasts showing continual growth. Small colony at top of photograph is degenerating. Fig. 19, 14 days; and Fig. 20, 16 days after explantation. $\times 25$.

FIG. 21. Higher magnification of blocked area in Fig. 19, showing protoplasmic processes. $\times 90$.

FIG. 22. Group of macrophages 5 days after explantation showing variation in size and shape. $\times 90$.

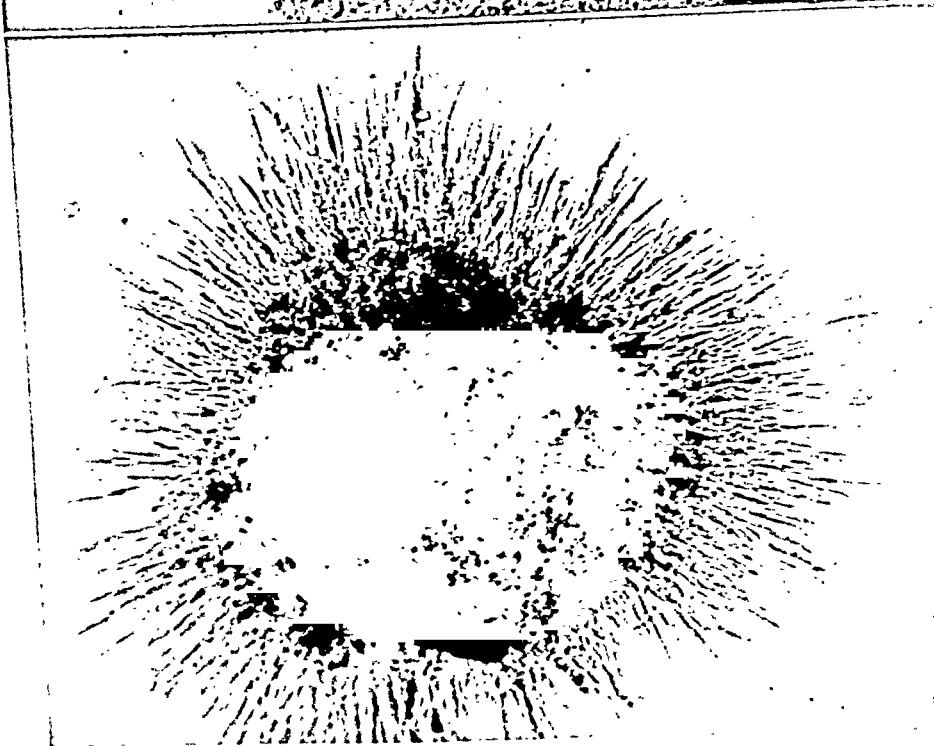
FIG. 23. Branching type of fibroblastic growth in colony 15 days after explantation. $\times 40$.

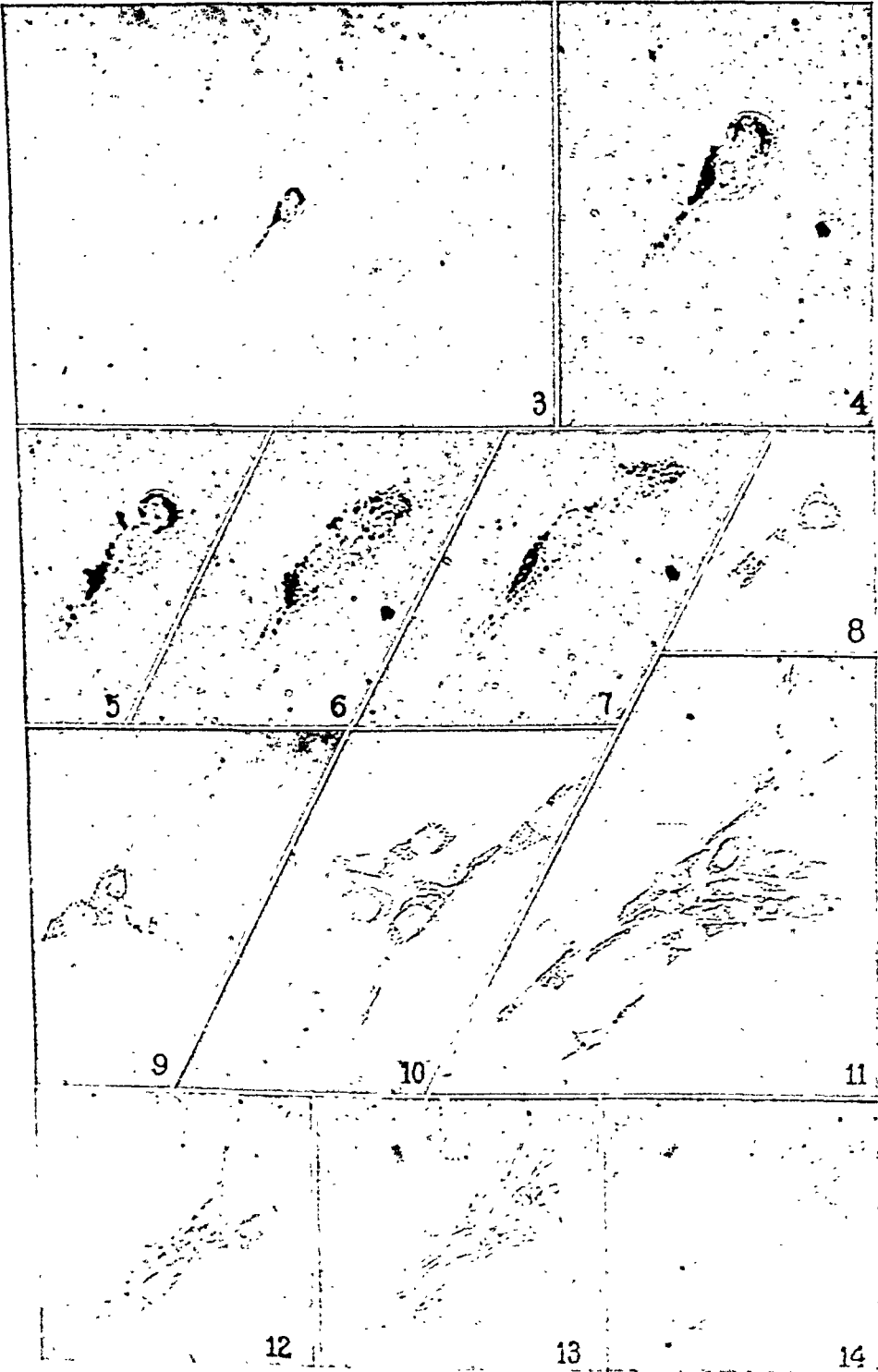
FIG. 24. "Transitional" type of cellular growth originating from single cell 9 days after explantation. Note the absence of pointed protoplasmic processes and the presence of peripheral zone of clear cytoplasm. $\times 90$.

FIG. 25. Fibroblastic cellular type 4 days after the second transplantation of fibroblastic growth from mononuclear exudative cells. $\times 90$.

FIG. 26. Growth from a large clump of mononuclear exudative cells 2 days after explantation showing macrophages and early fibroblastic growth. $\times 90$.

FIG. 27. Colony of fibroblasts from single cell 18 days after explantation showing degenerative changes. $\times 90$.





Micro Cultures from a single type of cell

BLOOD PLASMA PROTEIN REGENERATION CONTROLLED BY DIET

SYSTEMATIC STANDARDIZATION OF FOOD PROTEINS FOR POTENCY IN PROTEIN REGENERATION. FASTING AND IRON FEEDING

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One may properly ask three questions relating to plasma proteins. Where are these proteins formed? The finger of suspicion points to the liver although the proof is not yet absolute. From what materials are these proteins fabricated? Evidence given below shows that some food proteins are more potent than others in the regeneration of plasma proteins but one cannot group proteins into vegetable, grain, animal, complete or incomplete and relate their potency to such grouping. Each food protein must be tested and its potency measured. It will be of some interest to determine whether there are any key amino acids which are essential to this upbuilding of plasma protein. Are these plasma proteins static and only to be replaced when lost (hemorrhage, ascites, nephrosis) or are they dynamic and concerned with protein metabolism? This last question is answered at least in part in the second paper below in favor of an ebb and flow between plasma, organ and tissue protein—a dynamic concept.

Much evidence has been published relating to the potency of food factors for potency in the regeneration of new hemoglobin in experimental anemia in dogs (11). We note the widest range of potency in hemoglobin regeneration from a maximum due to a diet of liver to a minimum due to grains and fish. When it was found that plasma protein regeneration (3) like hemoglobin regeneration could be controlled by diet our first thought was that the food potencies for hemo-

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globin and plasma protein regeneration might run along parallel to each other. Like most "first thoughts" this was wrong. *Liver* to be sure was potent in both lists but *kidney* which is almost as potent as liver to regenerate new hemoglobin was found to be at the very bottom of the list of foods so far tested for the regeneration of new plasma proteins.

Beef serum stands at the head of our list of potent foods for new plasma protein regeneration. This perhaps is not surprising although hemoglobin when fed is poorly utilized for hemoglobin production in anemia. Feeding 2.6 gm. of *beef serum* protein will produce 1 gm. of new plasma protein. Under similar conditions to produce 1 gm. of new plasma protein we must feed about 20 gm. of kidney protein.

The general plan of these experiments is very simple although technical difficulties must be overcome by experience. The normal plasma protein level (6-7 per cent) is reduced by bleeding with return of washed red cells in Locke's solution (plasmapheresis) to plasma protein levels of 3.6-3.9 per cent which are just above the edema level for the dog under the conditions of these experiments. This low level of plasma proteins is maintained a constant by suitable bleedings and red cell replacement. The subnormal level of plasma proteins presumably acts as a *maximal stimulus* for the production of new plasma proteins and unless the plasmapheresis is continued almost daily the new formed plasma protein will bring the plasma protein concentration promptly back to normal.

When the plasma protein depletion is begun the weekly output of new plasma protein is quite high and gradually declines to the *basal output* within 4-6 weeks. After this initial period the basal output will continue indefinitely at a reasonably constant figure provided the basal diet and other factors are kept uniform. The initial high output may exceed the basal output of plasma protein by 30-120 gm. Allowing for the drop in plasma protein concentration the amount of this protein is far beyond any method errors and is designated a *reserve store* of protein building material. This reserve store varies depending upon the diet factors of the preceding weeks and must always be completely exhausted before the basal output becomes fairly constant. It is possible if not probable that some of this reserve store is held in the liver (7). Hemoglobin regeneration experiments in anemia

will show a similar reserve store of hemoglobin building material but often in larger amounts and it requires longer periods of depletion to exhaust this reserve. It is possible that the body holds a general reserve store of protein or protein building material which on demand can be used to manufacture either plasma protein or hemoglobin or tissue protein as occasion may require. Experiments are in progress which may throw light upon this point.

In a recent communication (3) we have mentioned some of the earlier papers dealing with plasma protein regeneration. Some of the recent papers may be mentioned briefly. Moschowitz (9) reports clinical conditions associated with hypoproteinemia particularly nephrosis, colitis, ascites, and pernicious anemia. He gives an excellent review. Jones, Eaton, and White (5) produced edema in cats by a low protein diet together with excess fluid or salt intake or infection. Jürgens and Gebhardt (6) believe the liver and reticulo-endothelial system are the sources of plasma proteins but do not consider diet a factor except as a stimulus. Reimann, Medes, and Fisher (10) conclude that the liver is responsible for the elaboration of protein precursors of blood proteins which are incorporated in widely distributed cells. In their experiments on rabbits no data relating to diet are given.

Methods

The various methods used were described in considerable detail in a recent paper (3) and this need not be repeated. The basal ration consists of boiled peeled white potatoes, canned tomatoes, Post bran flakes, Karo corn syrup, and cod liver oil in amounts as given in each dog's clinical history. Each dog received daily 1 gm. of a salt mixture (8) added to the diet. The potato contains 2.2 gm. protein per 100 gm. and the bran flakes contain 15 gm. protein per 100 gm.

The various food substances as used from time to time in animal experiments were analyzed for nitrogen in triplicate or quadruplicate by the macro-Kjeldahl method. The protein content was calculated as 6.25 times the average nitrogen values. The food substances were added to the basal ration and fed as a hash. The kidney tissue was cooked in water and fed with the broth. Egg white was coagulated by boiling. Other meats were fed raw. Commercial canned salmon was used. The serum was obtained from defibrinated fresh beef blood, the red cells being removed by use of the centrifuge. The fresh serum was mixed with the basal diet.

Nitrogen loss (negative N balance) included the urinary N plus 1 gm. daily allowed for the feces (not measured in all cases but calculated from average figures) plus the nitrogen contained in blood plasma removed by plasmapheresis and hematocrit samples.

EXPERIMENTAL OBSERVATIONS

All tabulated experiments are of the same character and the tables present continuous periods of observation divided into 7 day units. Rarely a period may be 6 or 8 days or longer but in the tables the values are corrected as of 7 days. Each dog has at least two tables to give complete experimental data. Tables 1, 2,

TABLE 1

*Blood Plasma Depletion and Regeneration
Kidney and Pancreas Compared with Beef Heart*

Dog 32-394.

Period 7 days	Diet	Protein intake Total for 7 days	Protein removed			Protein removed above basal*	Potency ratio* Protein in- take to pro- tein output	Blood plasma Average concen- tration	
			Albu- min	Glob- ulin	Total			Total protein	A/G ratio
		gm.	gm.	gm.	gm.	gm.		gm. per cent	
1	Basal	98			44.7			4.57	
2	Basal	98			30.5			3.80	0.62
3	Basal	98			28.9			3.57	
4	Basal	98			26.4			3.36	
5	Salmon bread	210	7.9	26.8	34.7	49.9	4.2	3.85	0.30
6	Basal	98	11.6	21.5	33.1			3.74	0.54
7	Basal	98	9.0	17.1	26.1			3.69	0.52
8	Gelatin	?			21.4			3.78	
9	Albumin	?	3.1	8.9	12.0			3.50	0.35
10	Basal	98	8.8	17.8	26.6			3.77	0.50
11	Basal	98	3.1	8.0	11.1			3.18	0.39
12	Albumin	293	10.0	10.9	20.9			3.48	0.92
13	Basal	98	5.4	11.7	17.1			3.47	0.46
14	Kidney	265	13.6	19.4	33.0	9.4	20.8	3.97	0.70
15	Basal	98	7.2	16.2	23.4			3.62	0.44
16	Basal	98	6.6	12.4	19.0			3.40	0.53
17	Pancreas	578	16.4	21.0	37.4	30.0	19.0	4.10	0.79
18	Basal	98	16.3	19.1	35.4			3.72	0.85
19	Basal	98	11.0	12.2	23.2			3.50	0.90
20	Beef heart	481	23.2	22.0	45.2	47.9	8.0	4.07	1.05
21	Basal	98	24.6	19.8	44.4			3.82	1.24
22	Basal	98	11.3	13.0	24.3			3.49	0.87

* Estimated basal output equivalent to 22 gm. plasma protein per week.

3, and 4 give the necessary figures for complete understanding of the plasma protein regeneration and the related diet potency. Tables 1-a, 2-a, etc., give the figures on nitrogen balance, nutrition, and blood findings. Following the respective tables is found the clinical history of each dog.

Tables 1 and 2 present a continuous history lasting over 41 weeks during which period the plasma protein level was held at 3.5-3.9 per cent by frequent plasmaphereses (4-7 per week). The low initial blood plasma protein level of 4.57 per cent is explained by a 2 months' preliminary period on the basal ration which contains no animal protein.

TABLE 1-a
Nitrogen Balance, Blood Findings, and Clinical Condition

Dog 32-394.

Period 7 days	Diet	Diet sup- plement per period	Weight	Negative N balance	Urinary N	R. B. C. hematocrit	Plasma volume
		gm.	kg.	gm.	gm.	per cent	cc.
			13.5			36.6	602
			13.8			39.8	
			13.6			41.7	
1	Basal		13.7	8.7	13.2	37.3	
2	Basal		13.4	0.8	21.8	35.2	
3	Basal		13.3	8.4	11.8	34.7	475
4	Basal	2100	13.2	22.4		41.6	
5	Salmon bread		13.2			40.3	720
6	Basal		13.2			40.8	
7	Basal	?	13.3			33.0	
8	Gelatin	?	12.7			39.4	594
9	Albumin		12.7		1.7	35.6	562
10	Basal		12.7		+26.1	10.5	541
11	Basal	250	12.4		5.9	10.6	656
12	Albumin		12.6		+12.9	16.7	550
13	Basal	1283	12.6		5.0	10.0	557
14	Kidney		12.5		18.7	39.9	665
15	Basal		12.9		+43.2	34.3	619
16	Basal	1360	12.9		12.0	15.0	724
17	Pancreas		13.2		7.2	12.2	770
18	Basal		13.0		+30.3	32.5	696
19	Basal	2100	13.7		14.2	15.8	683
20	Beef heart		14.1		10.3	15.1	
21	Basal		13.3				
22	Basal						

The estimated basal output for Tables 1 and 2 is given as 22 gm. plasma protein per week while the dog is on the basal ration after the reserve store has been exhausted. This is somewhat of an arbitrary figure but in all dogs so far tested it amounts to about 2 gm. plasma protein per kilo per week. One does not accept the lowest basal output for any given week but takes a general average of a number of

EXPERIMENTAL OBSERVATIONS

All tabulated experiments are of the same character and the tables present continuous periods of observation divided into 7 day units. Rarely a period may be 6 or 8 days or longer but in the tables the values are corrected as of 7 days. Each dog has at least two tables to give complete experimental data. Tables 1, 2,

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			Albu- min	Glob- ulin	Total			Total protein	A/G ratio
		gm.	gm.	gm.	gm.	gm.		gm. per cent	
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22	Basal	98	11.3	13.0	24.3			3.49	0.87

* Estimated basal output equivalent to 22 gm. plasma protein per week.

3, and 4 give the necessary figures for complete understanding of the plasma protein regeneration and the related diet potency. Tables 1-a, 2-a, etc., give the figures on nitrogen balance, nutrition, and blood findings. Following the respective tables is found the clinical history of each dog.

periods as noted in the tables where the protein or food intake is not given. The basal ration amounted to 525 gm. per day or about 66 calories per kilo.

It has been noted (3) that *albumin* may make up about half of the total plasma protein contributed from the reserve store and this figure will vary with the diet which precedes the depletion period. It will be observed in all the tables that the *globulin* makes up a considerable part of the total protein removed during basal control periods in which no animal proteins are fed—the usual ratio is 2 parts globulin to 1 part albumin. It was suggested (3) that vegetable and grain proteins might favor the production of globulin in contrast to animal protein which might favor albumin production. The *salmon bread* period (Table 1) gives some evidence to indicate that grain protein may favor globulin production. During the week of salmon bread feeding there was removed 7.9 gm. albumin and 26.8 gm. globulin which is associated with the lowest A/G ratio observed (0.30). The bulk of protein (about 60 per cent) in the salmon bread is from wheat. Salmon bread (12) contains wheat flour, potato starch, bran, sugar, tomatoes, cod liver oil, canned salmon, yeast, and a salt mixture and its preparation has been carefully described. Salmon bread in anemia will permit a minimum of hemoglobin regeneration but with plasma depletion it will produce abundant globulin and some albumin with a potency ratio of 4.2 to 1. The potency ratio for salmon bread is of necessity figured somewhat differently as the salmon bread *replaced* the basal diet. Period 5, Table 1, shows an intake of 210 gm. protein in the salmon bread. The plasma protein output in period 5 should be all credited to the salmon bread diet = $34.7 \text{ gm.} + 22 = 4.1$. Total in periods 6 and 7 or $33.1 - 22 = 11.1$ and $26.1 - 22 = 4.1$. Total credit to the salmon bread diet is 49.9 on an intake of 210 gm., a potency ratio of 4.2. This corresponds closely to the potency ratio of the basal diet which for this dog is 4.5—that is the basal diet protein intake (98 gm.) divided by the basal protein output (22 gm.). The potency ratio for salmon is given below (Table 4) as 15.2 which compares unfavorably with these grain proteins and beef muscle proteins. Albumen (Table 1, period 12) represents a feeding of "Egg Albumen, Impalpable Powder, Soluble, Merck." It is probable that most of

satisfactory low basal weeks where no heavy protein feeding has shortly preceded. If we averaged weeks 8-13 inclusive, the basal output would stand at 18 gm. plasma protein per week. However in two of these periods the dog was vomiting some of its diet and was clinically somewhat disturbed with some loss of weight. In view of the very low output of plasma protein during fasting (Table 4) it seems fair to place the *basal output* at 22 gm. Even if this figure is a gram or so too high it is on the conservative side and the potency ratio will be a bit larger.

The *potency ratio* means the grams of protein which must be fed to produce 1 gm. of new plasma protein above the basal output. Obviously the lower the ratio the more potent is the given diet factor (Table 1)—kidney protein (20 gm.) or beef heart protein (8 gm.) will each produce 1 gm. new plasma protein. In the tables this *potency ratio* is calculated from the total protein intake minus the basal protein intake divided by the total plasma protein removed over and above the basal plasma protein output per week.

When a potent diet factor is added to the basal ration after a control period there is usually no change for 2 days but on the 3rd day of feeding the plasma proteins begin to rise and call for larger and more frequent plasmaphereses to maintain the usual depletion level of plasma protein concentration. The plasma protein output reaches a peak on the 7th day of feeding when the dog is returned to the basal ration. The accelerated plasma protein output continues for many days depending upon the potency and amount of the diet factor but sinks gradually to the basal level, usually by the end of the 2nd week of the basal ration. We assume that material has been accumulated and the stored reserve again built up during the favorable diet period and this store is called out and finally exhausted during the after period of 2 weeks. This we term the "carry over" and the picture is like that described for the regeneration of new hemoglobin in anemia on a favorable diet.

The *reserve store* of this dog (Table 1) is found to total 43 gm. plasma protein which represents the total output (131 gm.) for the first 4 weeks minus 88 gm., the standard basal output for this period. This dog consumed 100 per cent of its rations at all times except in three

periods as noted in the tables where the protein or food intake is not given. The basal ration amounted to 525 gm. per day or about 66 calories per kilo.

It has been noted (3) that *albumin* may make up about half of the total plasma protein contributed from the reserve store and this figure will vary with the diet which precedes the depletion period. It will be observed in all the tables that the *globulin* makes up a considerable part of the total protein removed during basal control periods in which no animal proteins are fed—the usual ratio is 2 parts globulin to 1 part albumin. It was suggested (3) that vegetable and grain proteins might favor the production of globulin in contrast to animal protein which might favor albumin production. The *salmon bread* period (Table 1) gives some evidence to indicate that grain protein may favor globulin production. During the week of salmon bread feeding there was removed 7.9 gm. albumin and 26.8 gm. globulin which is associated with the lowest A/G ratio observed (0.30). The bulk of protein (about 60 per cent) in the salmon bread is from wheat. Salmon bread (12) contains wheat flour, potato starch, bran, sugar, tomatoes, cod liver oil, canned salmon, yeast, and a salt mixture and its preparation has been carefully described. Salmon bread in anemia will permit a minimum of hemoglobin regeneration but with plasma depletion it will produce abundant globulin and some albumin with a potency ratio of 4.2 to 1. The potency ratio for salmon bread is of necessity figured somewhat differently as the salmon bread *replaced* the basal diet. Period 5, Table 1, shows an intake of 210 gm. protein in the salmon bread. The plasma protein output in period 5 should be all credited to the salmon bread diet = 34.7 gm. plus the "carry over" in periods 6 and 7 or $33.1 - 22 = 11.1$ and $26.1 - 22 = 4.1$. Total credit to the salmon bread diet is 49.9 on an intake of 210 gm., a potency ratio of 4.2. This corresponds closely to the potency ratio of the basal diet which for this dog is 4.5—that is the basal diet protein intake (98 gm.) divided by the basal protein output (22 gm.). The potency ratio for salmon is given below (Table 4) as 15.2 which compares unfavorably with these grain proteins and beef muscle proteins.

Albumen (Table 1, period 12) represents a feeding of "Egg Albumen, Impalpable Powder, Soluble, Merck." It is probable that most of

this material was not absorbed or digested as there is no increase in the urinary N. There is a slight increase of the albumin plasma fraction at the expense of the globulin—compare Table 3 below.

Kidney (Table 1, period 14) presented a great surprise because this tissue is so potent in building new hemoglobin in anemia. Kidney shows the lowest potency ratio 20.8 and we must think of 21 gm. of kidney as required to form 1 gm. new plasma protein. It pushes the A/G ratio up toward unity but there is no comparison with beef heart in the same dog where the potency ratio is 8 and the A/G ratio 1.24.

Pancreas (Table 1, period 17) stands close to the kidney and its potency ratio is 19.0. It would appear that nucleoproteins which are abundant in the pancreas are not particularly concerned with plasma protein regeneration.

Beef heart (Table 1, period 20) is a meat protein much used in laboratory studies. Its potency ratio is 8.0 and it corresponds with liver in this respect. Beef heart however produces a very large output of albumin and pushes the A/G ratio well above unity (1.24). In this particular dog (Tables 1 and 2) beef heart shows the highest output of albumin, even more than skeletal muscle and gizzard (smooth muscle) both given in Table 2. This question of the specificity of diet proteins for the production of plasma albumin in more abundance than plasma globulin deserves further study.

Blood volume figures deserve some attention in this dog (Tables 1-a and 2-a). With animal protein feeding there is always an increase (100 cc. average) in plasma volume. One notes also in spite of heavy withdrawal that the plasma protein concentration rises above 4.0 per cent. These two facts would point to a considerable outpouring of new protein into the circulation and an actual volume response as well.

Red cell hematocrit figures in this dog are uniformly satisfactory and average about 35–40 per cent only somewhat below the normal of 50 per cent for the dog. Iron feeding shows a rise of red cell hematocrit to 50 per cent but this is a familiar response.

Weight curve in Tables 1-a and 2-a is satisfactory and indicates good nutrition and adequate diet intake. The urinary nitrogen figures are a bit irregular but are to be explained in part on the basis of urine retained in the bladder. These dogs are never catheterized and their urination is irregular.

Egg white is well utilized and shows a potency ratio of 5.8 which is practically identical with lactalbumin, gizzard, and skeletal muscle (Table 2). Egg white feeding causes almost as much albumin as globulin production (period 23, Table 2).

Liver extract (Table 2, period 26) indicates a combination of two liver fractions¹—No. 55 (13) which contains much of the material effective in hemoglobin regeneration in experimental hemorrhagic anemia in dogs and No. 343 which contains much of the material effective in the therapy of pernicious anemia. This material has been described elsewhere (13) and both fractions amount to 7 per cent of the fresh liver weight. Its potency for new hemoglobin production is very great and corresponds to 300 gm. fresh liver given daily for a week. Its potency for the regeneration of new plasma protein is negligible and corresponds to the small amount of nitrogenous material which the two fractions contain. The amount of new plasma protein is only 7.7 gm. above the basal level but as the calculated protein in the liver fraction fed amounts to only 67 gm. the potency ratio is 8.6 which is close to the potency ratio (6.5) for whole liver (Table 3).

Lactalbumin (Table 2, period 28) gives a liberal response and we note that a good deal more globulin than albumin is produced so that the A/G ratio does not rise above 0.72. The potency ratio is 5.5 and obviously the material is completely digested and utilized.

Ferric citrate fed in large amounts (2 gm. daily) gives a reaction which is distinct but not adequately explained. This dose of 2 gm. corresponds to 360 mg. of Fe which in an anemic dog will give a maximal regeneration of hemoglobin, approximately 50 gm. new hemoglobin from 1 week iron administration. This dog put out 14.4 gm. new plasma protein above the basal level. The same dog at this time had a plasma volume of 700 cc. and a protein concentration of 3.76 per cent or a circulating mass of plasma protein of 26 gm. Iron administration caused the production of plasma protein amounting to 55 per cent of the total proteins in the circulating plasma. This amount is far beyond any possible experimental error or known physiological fluctuation. The proper explanation will be of considerable significance.

Gizzard (Table 2, period 36) means that the smooth muscle only was

¹ Valuable material was supplied by Eli Lilly and Company.

fed and the lining mucosa with the tendinous parts were cut away. Its reaction is very much like the skeletal muscle response and the potency ratios are about the same.

TABLE 2
Blood Plasma Depletion and Regeneration
Lactalbumin, Gizzard, and Striated Muscle All Potent
Iron Reaction Unexplained

Dog 32-394.

Period 7 days	Diet	Protein intake Total for 7 days	Protein removed			Protein removed above basal*	Potency ratio* Protein in- take to pro- tein output	Blood plasma Average concen- tration	
			Albu- min	Glob- ulin	Total			Total protein	A/G ratio
		gm.	gm.	gm.	gm.	gm.		gm. per cent	
23	Egg white	295	18.7	23.7	42.4	33.5	5.8	3.87	0.78
24	Basal	98	16.7	17.6	34.3			3.65	0.95
25	Basal	98	8.9	13.9	22.8			3.53	0.64
26	Liver extract	165	10.3	17.8	28.1	7.7	8.6	3.57	0.58
27	Basal	98	7.8	15.8	23.6			3.61	0.49
28	Lactalbumin	389	18.1	25.1	43.2	52.5	5.5	4.17	0.72
29	Basal	98	15.8	23.5	39.3			3.61	0.67
30	Basal	98	11.3	20.7	32.0			3.68	0.55
31	Basal	98	10.9	15.1	26.0			3.67	0.72
32	Ferric citrate	98	10.7	20.3	31.0	14.4		3.76	0.53
33	Basal	98	7.1	20.3	27.4			3.87	0.35
34	Liver residue	?			26.1			3.95	
35	Basal	98	8.2	13.8	22.0			3.43	0.59
36	Gizzard	408	22.1	27.8	49.9	58.5	5.3	4.10	0.79
37	Basal	98	20.9	21.7	42.6			3.78	0.96
38	Basal	98	12.3	19.7	32.0			3.66	0.62
39	Skeletal muscle	475	25.5	31.2	56.7	66.4	5.7	4.17	0.82
40	Basal	98	17.6	20.5	38.1			3.96	0.86
41	Basal	98	13.5	24.1	37.6			3.70	0.56

* Estimated basal output equivalent to 22 gm. plasma protein per week.

Skeletal muscle (Table 2, period 39) shows an active production of albumin as well as globulin and the A/G ratio comes back toward normal. It may be argued properly that the gizzard and skeletal muscle periods should have been followed by 3 weeks basal diet to exhaust completely the "carry over." This probably would give potency ratios slightly smaller than those recorded.

Clinical History, Dog 32-394.—An adult, female, bull mongrel weighing 13 kg. This dog had been on basal diet for 2 months before depletion was begun. The basal daily diet consisted of 300 gm. white potato (6.6 gm. protein); 100 gm. tomato; 50 gm. Post's bran flakes (7.5 gm. protein); 50 gm. Karo syrup; 25 cc. cod liver oil; and 1 gm. salt mixture. The plasma protein level following this basal period was 4.82 per cent, albumin 1.76 per cent, and globulin 3.05 per cent. The blood volume was 920 cc., the plasma volume 560 cc. From periods 1 to 41 the

TABLE 2-a

Nitrogen Balance, Blood Findings, and Clinical Condition

Dog 32-394.

Period 7 days	Diet	Diet sup- plement per period	Weight	Negative N balance	Urinary N	R. B. C. hematocrit	Plasma volume
		gm.	kg.	gm.	gm.	per cent	cc.
23	Cooked egg white	1750	13.6	+7.7	25.7	37.8	745
24	Basal		13.4	16.1	19.3	40.5	753
25	Basal		13.0	12.6	17.6	38.6	682
26	Liver extract	150	13.0	2.7	17.6	44.8	557
27	Basal		13.3	9.0	13.9	44.4	582
28	Lactalbumin	350	13.4	+25.9	21.2	45.6	721
29	Basal		13.7	16.2	18.6	44.2	695
30	Basal		13.4	11.6	15.2	36.7	685
31	Basal		13.1	13.2	17.7	40.1	647
32	Ferric citrate	14	12.9	11.6	15.3	47.5	707
33	Basal		12.8	9.6	13.9	51.2	559
34	Liver residue	?	12.5			46.5	
35	Basal		12.4			42.8	408
36	Gizzard	1400	12.7	+24.7	25.6	40.2	625
37	Basal		13.1	13.6	15.5	41.9	580
38	Basal		13.1	12.5	16.1	43.7	565
39	Skeletal muscle	1400	13.3	+35.0	25.9	38.1	647
40	Basal		13.7	16.4	19.0	44.6	629
41	Basal		13.4	15.5	18.0	47.3	627

non-protein nitrogen of the blood varied from 12 mg. to 30 mg. per cent, reaching the latter level only once and that during period 40. During the weeks of initial depletion (periods 1-4), the dog maintained its body weight and remained in excellent clinical condition. During period 5 the dog was given salmon bread 300 gm. daily to replace the basal ration. During period 8 the dog refused gelatin mixed with the basal diet, and vomited the gelatin when it was given separately by stomach tube. On this account the gelatin feeding was discontinued after 4 days. During the following 8 days the dog was fed the basal ration and consumed 100 per cent of the diet. This 12 day period is recorded as a 7 day period, the

proper correction being made. In the last 3 days of period 9 the dog became lethargic, refused food, and developed hemoglobinuria. The attempt to feed powdered egg albumen was discontinued and the dog put back on the basal ration. Dextrose was given by stomach tube. During the two following periods on the basal ration (periods 10-11), the dog was again in good health, the hemoglobinuria had ceased, the dog was active, and consumed all food. Periods 10-33 were without mishap. The diet ration in each particular experiment during that time was consumed 100 per cent. Liver residue feeding was attempted during the first

TABLE 3
Blood Plasma Depletion and Regeneration
Liver and Serum Potent Factors

Dog 32-130.

Period 7 days	Diet	Protein intake Total for 7 days	Protein removed			Protein removed above basal*	Potency ratio* Protein in- take to pro- tein output	Blood plasma Average concen- tration	
			Albu- min	Glob- ulin	Total			Total protein	A/G ratio
		gm.	gm.	gm.	gm.	gm.		gm. per cent	
1	Basal	114	22.6	14.3	36.9			5.34	1.58
2	Basal	114	16.6	23.3	39.9			4.28	0.71
3	Basal	114	10.9	18.9	29.8			3.99	0.58
4	Basal	114	14.2	19.9	34.1			3.89	0.71
5	Albumin	407	12.4	18.5	30.9	?	?	3.78	0.67
6	Basal	114	12.9	19.7	32.6			3.80	0.65
7	Basal	114	8.3	17.6	25.9			3.57	0.47
8	Liver	534	25.2	29.6	54.8	65.2	6.5	4.44	0.85
9	Basal	114	27.2	31.8	59.0			3.99	0.85
10	Basal	114	13.6	18.8	32.4			3.79	0.72
11	Ox serum	335	28.3	26.1	54.4	83.0	2.6	3.97	1.08
12	Basal	114	32.1	33.4	65.5			3.98	0.96
13	Basal	114	18.7	25.4	44.1			3.83	0.74
14	Skeletal muscle	533	34.9	39.2	74.1	47+		4.53	0.89

* Estimated basal output equivalent to 27 gm. plasma protein per week.

2 days of period 34. The dog refused the residue mixed with the basal diet, and vomited the mixture when it was spoon fed. During the remaining 5 days of this period the basal ration was fed, which was entirely consumed. Periods 35-41 were uneventful. During the 41 weeks 55.58 liters of blood were removed in 202 exchanges (plasmaphereses). Following period 41 beef serum concentrated by drying was given. After 3 days of this feeding, the dog refused food and developed hemoglobinuria and marked conjunctivitis. Petechiae were present on the mucous membranes. The sclerae were icteric. The dog was returned to the

basal ration and given blood transfusions and dextrose by vein. Three days later the animal was found dead. Autopsy revealed: Central necroses of liver involving perhaps two-thirds of the liver substance. Hemorrhagic bronchopneumonia. Acute bacterial endocarditis, mitral valve. Hemorrhagic bronchopneumonia. Acute focal nephritis. Hemorrhage into large bowel. Petechiae on mucous membranes and pleurae. Microscopical sections showed considerable iron containing pigment in spleen, liver, kidneys, lymph nodes, and bone marrow.

Beef serum and liver are adequately standardized in this dog (Table 3, periods 8 and 11). Beef serum stands at the head of the list of

TABLE 3-a
Nitrogen Balance, Blood Findings, and Clinical Condition
Dog 32-130.

Period 7 days	Diet	Diet sup- plement per period	Weight	Negative N balance	Urinary N	R. B. C. hematocrit	Plasma volume
		gm.	kg.	gm.	gm.	per cent	cc.
1	Basal	350	14.6			43.3	
2	Basal		14.2			34.1	
3	Basal		14.4	9.5	16.0	34.4	702
4	Basal		13.6	10.2	16.0	39.1	711
5	Albumen	2100	13.3		13.9	36.9	623
6	Basal		12.9	6.0	12.1	40.5	690
7	Basal		12.5	7.3	14.4	39.5	693
8	Liver		13.1	+33.8	35.8	37.9	755
9	Basal	2800	13.6	25.5	27.3	35.6	707
10	Basal		13.4	12.5	18.6	29.7	623
11	Ox serum		13.4	4.8	33.1	33.7	743
12	Basal		13.4	28.6	29.3	24.8	
13	Basal	2100	13.3	20.6	24.8	26.3	760
14	Skeletal muscle		13.7			25.2	

potent materials most favorable for new plasma protein regeneration. Beef plasma protein (2.6 gm.) will produce 1 gm. of new plasma protein in the depleted dog—a potency ratio of 2.6. Moreover there is a large output of albumin and the A/G ratio rises to 1.08. The A/G ratio of the beef serum as fed reads 1.07. It is probable that not all of the "carry over" was removed as the 2nd week on the basal diet shows a total protein output of 44 gm. (Table 3, period 13) and the total plasma protein level is 3.83 per cent. Had this experiment been continued through a third control week a little more of the "carry

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Liver and Serum Potent Factors

Dog 32-130.

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			Albu- min	Glob- ulin	Total			Total protein	A/G ratio
		gm.	gm.	gm.	gm.	gm.		gm. per cent	
1	Basal	114	22.6	14.3	36.9			5.34	1.58
2	Basal	114	16.6	23.3	39.9			4.28	0.71
3	Basal	114	10.9	18.9	29.8			3.99	0.58
4	Basal	114	14.2	19.9	34.1			3.89	0.71
5	Albumin	407	12.4	18.5	30.9	?	?	3.78	0.67
6	Basal	114	12.9	19.7	32.6			3.80	0.65
7	Basal	114	8.3	17.6	25.9			3.57	0.47
8	Liver	534	25.2	29.6	54.8	65.2	6.5	4.44	0.85
9	Basal	114	27.2	31.8	59.0			3.99	0.85
10	Basal	114	13.6	18.8	32.4			3.79	0.72
11	Ox serum	335	28.3	26.1	54.4	83.0	2.6	3.97	1.08
12	Basal	114	32.1	33.4	65.5			3.98	0.96
13	Basal	114	18.7	25.4	44.1			3.83	0.74
14	Skeletal muscle	533	34.9	39.2	74.1	47+		4.53	0.89

* Estimated basal output equivalent to 27 gm. plasma protein per week.

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Period 7 days	Diet	Diet sup- plement per period	Weight	Negative N balance	Urinary N	R. B. C. hematocrit	Plasma volume
		gm.	kg.	gm.	gm.	per cent	cc.
1	Basal		14.6			43.3	
2	Basal		14.2			34.1	
3	Basal		14.4	9.5	16.0	34.4	702
4	Basal		13.6	10.2	16.0	39.1	711
5	Albumen	350	13.3		13.9	36.9	623
6	Basal		12.9	6.0	12.1	40.5	690
7	Basal		12.5	7.3	14.4	39.5	693
8	Liver	2100	13.1	+33.8	35.8	37.9	755
9	Basal		13.6	25.5	27.3	35.6	707
10	Basal		13.4	12.5	18.6	29.7	623
11	Ox serum	2800	13.4	4.8	33.1	33.7	743
12	Basal		13.4	28.6	29.3	24.8	
13	Basal		13.3	20.6	24.8	26.3	760
14	Skeletal muscle	2100	13.7			25.2	

potent materials most favorable for new plasma protein regeneration. Beef plasma protein (2.6 gm.) will produce 1 gm. of new plasma protein in the depleted dog—a potency ratio of 2.6. Moreover there is a large output of albumin and the A/G ratio rises to 1.08. The A/G ratio of the beef serum as *fed* reads 1.07. It is probable that not all of the “carry over” was removed as the 2nd week on the basal diet shows a total protein output of 44 gm. (Table 3, period 13) and the total plasma protein level is 3.83 per cent. Had this experiment been continued through a third control week a little more of the “carry

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			Albu- min	Glob- ulin	Total			Total protein	A/G ratio
		gm.	gm.	gm.	gm.	gm.		gm. per cent	
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3	Basal	114	10.9	18.9	29.8			3.99	0.58
4	Basal	114	14.2	19.9	34.1			3.89	0.71
5	Albumin	407	12.4	18.5	30.9	?	?	3.78	0.67
6	Basal	114	12.9	19.7	32.6			3.80	0.65
7	Basal	114	8.3	17.6	25.9			3.57	0.47
8	Liver	534	25.2	29.6	54.8	65.2	6.5	4.44	0.85
9	Basal	114	27.2	31.8	59.0			3.99	0.85
10	Basal	114	13.6	18.8	32.4			3.79	0.72
11	Ox serum	335	28.3	26.1	54.4	83.0	2.6	3.97	1.08
12	Basal	114	32.1	33.4	65.5			3.98	0.96
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14	Skeletal muscle	533	34.9	39.2	74.1	47+		4.53	0.89

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						43.3	
			14.6			34.1	
			14.2			34.4	702
1	Basal		14.4	9.5	16.0	39.1	711
2	Basal		13.6	10.2	16.0	36.9	623
3	Basal	350	13.3		13.9	40.5	690
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8	Basal		13.4	12.5	18.6	33.7	743
9	Basal		13.4	4.8	33.1	24.8	
10	Basal	2800	13.4	28.6	29.3	26.3	
11	Ox serum		13.3	20.6	24.8	25.2	760
12	Basal		13.7				
13	Basal						
14	Skeletal muscle	2100					

potent materials most favorable for new plasma protein regeneration. Beef plasma protein (2.6 gm.) will produce 1 gm. of new plasma protein in the depleted dog—a potency ratio of 2.6. Moreover there is a large output of albumin and the A/G ratio rises to 1.08. The A/G ratio of the beef serum as fed reads 1.07. It is probable that not all of the “carry over” was removed as the 2nd week on the basal diet shows a total protein output of 44 gm. (Table 3, period 13) and the total plasma protein level is 3.83 per cent. Had this experiment been continued through a third control week a little more of the “carry

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		gm.	gm.	gm.	gm.	gm.		gm. per cent	
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2	Basal	114	16.6	23.3	39.9			4.28	0.71
3	Basal	114	10.9	18.9	29.8			3.99	0.58
4	Basal	114	14.2	19.9	34.1			3.89	0.71
5	Albumin	407	12.4	18.5	30.9	?	?	3.78	0.67
6	Basal	114	12.9	19.7	32.6			3.80	0.65
7	Basal	114	8.3	17.6	25.9			3.57	0.47
8	Liver	534	25.2	29.6	54.8	65.2	6.5	4.44	0.85
9	Basal	114	27.2	31.8	59.0			3.99	0.85
10	Basal	114	13.6	18.8	32.4			3.79	0.72
11	Ox serum	335	28.3	26.1	54.4	83.0	2.6	3.97	1.08
12	Basal	114	32.1	33.4	65.5			3.98	0.96
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14	Skeletal muscle	533	34.9	39.2	74.1	47+		4.53	0.89

* Estimated basal output equivalent to 27 gm. plasma protein per week.

2 days of period 34. The dog refused the residue mixed with the basal diet, and vomited the mixture when it was spoon fed. During the remaining 5 days of this period the basal ration was fed, which was entirely consumed. Periods 35-41 were uneventful. During the 41 weeks 55.58 liters of blood were removed in 202 exchanges (plasmaphereses). Following period 41 beef serum concentrated by drying was given. After 3 days of this feeding, the dog refused food and developed hemoglobinuria and marked conjunctivitis. Petechiae were present on the mucous membranes. The sclerae were icteric. The dog was returned to the

basal ration and given blood transfusions and dextrose by vein. Three days later the animal was found dead. Autopsy revealed: Central necroses of liver involving perhaps two-thirds of the liver substance. Hemorrhagic bronchopneumonia. Acute bacterial endocarditis, mitral valve. Hemorrhagic bronchopneumonia. into large bowel. Petechiae on mucous membranes and pleurae. Microscopical sections showed considerable iron containing pigment in spleen, liver, kidneys, lymph nodes, and bone marrow.

Beef serum and *liver* are adequately standardized in this dog (Table 3, periods 8 and 11). Beef serum stands at the head of the list of

TABLE 3-a
Nitrogen Balance, Blood Findings, and Clinical Condition
Dog 32-130.

Period days	Diet	Diet supplement per period gm.	Weight kg.	Negative N balance gm.	Urinary N gm.	R. B. C. hematocrit per cent	Plasma volume cc.
1	Basal	350	14.6			43.3	
2	Basal		14.2			34.1	
3	Basal		14.4	9.5	16.0	34.4	702
4	Basal		13.6	10.2	16.0	39.1	711
5	Albumen	2100	13.3		13.9	36.9	623
6	Basal		12.9	6.0	12.1	40.5	690
7	Basal		12.5	7.3	14.4	39.5	693
8	Liver		12.5	+33.8	35.8	37.9	755
9	Basal	2800	13.1		27.3	35.6	707
10	Basal		13.6	25.5	18.6	35.6	623
11	Ox serum		13.4	4.8	33.1	29.7	743
12	Basal		13.4	28.6	29.3	33.7	
13	Basal	2100	13.4	20.6	24.8	24.8	760
14	Skeletal muscle		13.3			25.2	

potent materials most favorable for new plasma protein regeneration. Beef plasma protein (2.6 gm.) will produce 1 gm. of new plasma protein in the depleted dog—a potency ratio of 2.6. Moreover there is a large output of albumin and the A/G ratio rises to 1.08. The A/G ratio of the beef serum as fed reads 1.07. It is probable that not all of the "carry over" was removed as the 2nd week on the basal diet shows a total protein output of 44 gm. (Table 3, period 13) and the total plasma protein level is 3.83 per cent. Had this experiment been continued through a third control week a little more of the "carry

over" might have been removed which would place the potency ratio slightly below the recorded figure. A repetition of this experiment using smaller amounts of serum is to be desired.

It is of interest that 100 gm. of liver compare almost exactly with 100 cc. of beef serum when tested for their potency to regenerate new plasma protein. The liver has 2.5 times as much protein as the beef serum. The potency ratio for the liver is 6.5. The potency ratio for liver previously reported (3) was 6.8.

Albumen as used in Table 3, period 5, is a chemical preparation ("Egg Albumen, Impalpable Powder, Soluble, Merck") and appears to be inert physiologically.

The *basal output* for this dog was estimated to be 27 gm. plasma protein per week and the basal diet contained 114 gm. protein per week giving a potency ratio of 4.2 which compares with 4.5 for the basal diet in the preceding experiments (Tables 1 and 2).

The *reserve store* of protein building material amounted to 40 gm. estimating the basal output at 27 gm. plasma protein per week. It required 6 weeks to exhaust this reserve which was found to be highest on meat diets and lowest after long diet periods unfavorable to plasma protein regeneration. The kennel diet of hospital table scraps has a large factor of uncertainty but is more favorable as a rule for plasma protein regeneration than the basal ration as given this dog.

Table 3-*a* shows a slight loss of weight and a negative nitrogen balance. The red cell hematocrit fell below a safe figure and this may have been a factor in the terminal bronchopneumonia. The large plasmaphereses which equalled one-third the blood volume each day, during the last week were important in causing the hemoglobinuria which is always a danger sign.

Clinical History, Dog 32-130.—An adult male hound weighing 14.5 kg. was placed on a basal diet consisting of 400 gm. potato (8.8 gm. protein); 100 gm. tomato; 50 gm. Post's bran flakes (7.5 gm. protein); 50 gm. Karo syrup; 25 cc. cod liver oil; 1 gm. salt mixture; and plasmapheresis was started at once. During the preceding month the dog was on the kennel diet of hospital table scraps. The initial plasma protein level was 6.30 per cent; albumin 3.12 per cent; and globulin 3.18 per cent. The blood volume was 1215 cc., the plasma volume 638 cc. The non-protein nitrogen throughout the experiments varied from 13–24 mg. per cent.

The dog's clinical condition was excellent throughout the periods of observation until the 12th week when the red cell hematocrit ranged between 23 and 30 per cent. Two transfusions of red cells suspended in Locke's solution were given during this week. The clinical condition was otherwise quite good. In the 14th week however, the dog refused its diet and appeared to be very weak. The hematocrit was 23 per cent, rectal temperature 39.8°C. The plasma was quite red due to hemolysis and there was hemoglobinuria. Dextrose (100 cc. of a 10 per cent solution) was given by stomach tube and the animal's condition improved only slightly. The following day the dog was killed by gas anesthesia in order to study the pathological lesions. Autopsy showed: Bronchopneumonia. Central necroses of liver. Blood pigment in spleen, kidneys, and lymph glands. Chronic myocarditis.

Dog 33-238 (Table 4) is an extremely interesting animal. Even after 10 weeks on the basal ration without depletion, the A/G ratio was unity and during the 14 weeks of plasma depletion the dog produced surprisingly large amounts of albumin on the basal ration so that the A/G ratio was usually close to unity. Moreover the utilization of the food protein was unusually complete. The dog received 66 calories per kilo and 52 gm. protein each week—15.4 gm. protein from potato and 36.7 gm. protein from the bran flakes. The potency ratio for this diet is 2.7 or to produce 1 gm. plasma protein only 2.7 gm. protein in the given diet mixture need be fed. This is about as favorable a diet reaction as is seen with beef serum (Table 3). Whether the potato protein or bran protein is responsible we cannot say with certainty but the basal diets in which a larger protein proportion is fed as potato do not give as favorable a potency ratio. However these factors have not yet been tested on the same dog to eliminate individual metabolic capacities.

Salmon (a commercial canned product) has a low potency for regeneration of plasma protein—a potency ratio of 15.2. This test is wholly satisfactory in all respects and the 2nd basal diet week shows a return to the basal output (19 gm. protein).

Liver residue (Table 4, period 7) contains most of the liver protein and is the residue after extraction and removal of the fractions potent in anemia described above (Table 2, period 26). It is seen that the potency ratio for liver residue is 7.4 and for whole fresh liver is 6.5 (Table 3, period 8). Evidently the potency for this material resides largely in the protein fraction and is not much disturbed by the preparation methods—acid and heat.

Fasting (Table 4, periods 10 and 11) gives information of great interest. The experiment was wholly satisfactory and the dog was in excellent condition at all times. 60 gm. of dextrose in 200 cc. water were given daily by stomach tube. The fecal N was not determined but estimated from other similar experiments to be about 0.4 gm. per day. There was loss of 1.4 kilograms in weight and the usual

TABLE 4
Blood Plasma Depletion and Regeneration
Fish and Liver Residue Compared with Fasting

Dog 33-238.

Period 7 days	Diet	Protein intake Total for 7 days	Protein removed			Protein removed Above basal*	Potency ratio* Protein intake to protein output	Blood plasma Average concentration	
			Albu- min	Globu- lin	Total			Total protein	A/G ratio
		gm.	gm.	gm.	gm.	gm.		gm. per cent	
1	Basal	52	17.2	17.7	34.9			3.98	0.97
2	Basal	52	11.7	11.8	23.5			3.83	0.99
3	Basal	52	10.7	11.8	22.5			3.78	0.91
4	Salmon	331	13.8	16.7	30.5	18.3	15.2	4.03	0.83
5	Basal	52	12.1	13.3	25.4			3.75	0.91
6	Basal	52	10.1	9.3	19.4			3.66	1.08
7	Liver residue	222	16.1	15.4	31.5	23.1	7.4	3.95	1.05
8	Basal	52	13.2	16.7	29.9			3.66	0.79
9	Basal	52	7.1	11.6	18.7			3.58	0.61
10	Dextrose	0			3.5			3.45	1.00
11	Dextrose	0			5.4			3.84	0.67
12	Basal	52	8.5	10.6	19.1			3.43	0.80
13	Skeletal muscle	265	16.7	14.9	31.6			4.32	1.12
14	Basal	52			27.2			3.72	

* Estimated basal output equivalent to 19 gm. plasma protein per week.

shrinkage of plasma volume from 465-373 cc. The dog had been very carefully standardized and the basal plasma protein output (19 gm. per week) is observed in the week preceding and following the sugar periods. The total plasma protein output for these 2 weeks is 9 gm. About 3 gm. of this amount can be accounted for by the shrinkage of plasma volume so that the total output amounts to about 3 gm. per week. This is close to physiological limitations. Evidently this dog can produce little if any new plasma proteins

during fasting periods—a very different story from that relating to new hemoglobin production in anemia or new liver cell production after injury.

The low N output in the urine after the sugar periods (Table 4-a, period 12) is of interest and shows distinct conservation of protein products.

TABLE 4-a

Nitrogen Balance, Blood Findings, and Clinical Condition

Dog 33-238.

Period 7 days	Diet	Diet supple- ment per period	Weight	Negative N balance	Urinary N	R.B.C. hemato- crit	Plasma volume
		gm.	kg.	gm.	gm.	per cent	cc.
1	Basal		9.8			48.7	417
2	Basal		9.7			43.1	
3	Basal		9.7	18.9	16.6	47.3	
4	Salmon	1050	9.7	+15.8	25.3	48.5	451
5	Basal		9.8	17.8	15.1	51.5	413
6	Basal		9.6	13.9	12.1	53.5	
7	Liver residue	252	10.0	+5.4	18.2	55.3	398
8	Basal		11.1	15.9	12.4	51.0	416
9	Basal		9.9	11.6	9.9	51.0	465
10	Dextrose		9.3	11.0	7.6	44.7	452
11	Dextrose		8.5	9.6	5.9	43.3	373
12	Basal		9.2	9.3	7.6	48.1	353
13	Skeletal muscle	1050	9.9	+13.7	16.6	52.0	430
14	Basal		10.1		20.6		

Table 4-a shows a highly satisfactory condition. The weight is uniform except during the sugar feeding period. The red cell hematocrit is maintained at practically a normal level throughout. The food consumption was 100 per cent in all periods.

Clinical History, Dog 33-238.—An adult male terrier weighing 9.8 kg. This dog had been on the basal ration consisting of 100 gm. potato (2.2 gm. protein); 60 gm. tomato; 50 gm. Karo syrup; 35 gm. Post's bran flakes (5.25 gm. protein); 25 cc. cod liver oil; and 1 gm. salt mixture, for 10 weeks prior to the beginning of plasmapheresis. The *reserve store* in this dog amounts to only 24 gm. of potential protein building material. The protein level following this basal period was 4.5 per cent; albumin 2.26 per cent; globulin 2.25 per cent. The blood volume was 872 cc. and the plasma volume 417 cc. The non-protein nitrogen ranged between

15 and 20 mg. per cent throughout the periods of observation. The dog was in excellent clinical condition up to the day of its death which was due to a mistake in the plasmapheresis—a hypertonic solution having been used. Autopsy showed: Pulmonary edema and organs which were grossly normal but for congestion. Microscopical sections of organs showed normal tissues. There was a slight excess of brown pigment in the spleen pulp and Kupffer cells of the liver.

Table 5 shows all the potency ratios for comparison. The *potency ratio* means the number of grams as protein which must be fed to re-

TABLE 5
Summary of Diets and Potency Ratios

Dietary factors	D/P or potency ratio		
	Dog 32-394	Dog 32-130	Dog 33-238
Beef serum.....		2.6	
Basal ration.....	4.5	4.2	2.7
Salmon bread.....	4.2		
Gizzard.....	5.3		
Lactalbumin.....	5.5		
Skeletal muscle.....	5.7		
Egg white.....	5.8		
Liver.....		6.5	
Liver residue.....			7.4
Liver extract.....	8.6		
Beef heart.....	8.0		
Salmon.....			15.2
Pancreas.....	19.0		
Kidney.....	20.8		

generate 1 gm. of plasma protein in depleted standard dog. It may be called the D/P ratio—diet to plasma protein.

DISCUSSION

The unexpected reaction of these dogs with plasma protein depletion to fasting and iron feeding calls for discussion. *Fasting* during anemia periods has been studied in this laboratory and in a recent report (1) it was shown that the dog can produce a good deal of new hemoglobin during fasting or sugar feeding periods and the reaction is striking if large doses of iron are given with the sugar. The evidence points to *conservation* of nitrogenous end products which are built up into new

hemoglobin. Furthermore during sugar feeding periods the dog can regenerate a considerable mass of new liver cells to repair a liver injury caused by chloroform (2). But in a similar emergency with depleted plasma proteins the dog can produce little if any new plasma protein.

We cannot say that one of these substances is any more urgently needed than the other. Moreover there is much evidence that plasma protein when present in excess can be utilized in the body to replace other proteins (4). With plasma depletion to levels below 4.0 per cent it is apparent that there is a strong stimulus to form new plasma protein from any satisfactory diet material. Moreover when the dog (Table 4, period 12) is put back on the basal ration the production of new plasma proteins begins immediately and progresses at the usual pace showing the normal basal output for that week. One might anticipate a subnormal plasma protein output owing to the demand coming from other depleted body stores of protein. There is a conspicuously low figure for urinary N on this first week after fasting, indicating very careful conservation of all nitrogenous material. There is a gain in weight of 0.7 kg.

We may state that in an emergency the plasma protein may contribute to body protein but the current will not flow easily in the opposite direction and the body proteins may be said to stand by helpless to aid while vital plasma proteins are depleted even to a lethal point. This is the only possible conclusion to be drawn from Table 4 but repeated observations must determine whether this is a constant reaction. Evidently in this emergency the plasma protein is very largely if not wholly dependent on materials coming in from the gastro-intestinal tract. Here again the finger of suspicion is placed on the liver, as these materials must be assembled into the complex structure of albumin or globulin.

The *reserve store* of plasma protein building material represents insurance for the normal dog against dangerous depletion of plasma proteins during a fasting period. This reserve store may amount to 30-120 gm. of potential plasma protein material—twice or three times as much on the average as this type of dog has in its circulation. We may well ask as to the nature of this *reserve store*. Although some of it may be in mature protein form the inability of body proteins during fasting to contribute to the plasma proteins speaks against this and

15 and 20 mg. per cent throughout the periods of observation. The dog was in excellent clinical condition up to the day of its death which was due to a mistake in the plasmapheresis—a hypertonic solution having been used. Autopsy showed: Pulmonary edema and organs which were grossly normal but for congestion. Microscopical sections of organs showed normal tissues. There was a slight excess of brown pigment in the spleen pulp and Kupffer cells of the liver.

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Kidney.....	20.8		

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hemoglobin. Furthermore during sugar feeding periods the dog can regenerate a considerable mass of new liver cells to repair a liver injury caused by chloroform (2). But in a similar emergency with depleted plasma proteins the dog can produce little if any new plasma protein.

We cannot say that one of these substances is any more urgently needed than the other. Moreover there is much evidence that plasma protein when present in excess can be utilized in the body to replace other proteins (4). With plasma depletion to levels below 4.0 per cent it is apparent that there is a strong stimulus to form new plasma protein from any satisfactory diet material. Moreover when the dog (Table 4, period 12) is put back on the basal ration the production of new plasma proteins begins immediately and progresses at the usual pace showing the normal basal output for that week. One might anticipate a subnormal plasma protein output owing to the demand coming from other depleted body stores of protein. There is a conspicuously low figure for urinary N on this first week after fasting, indicating very careful conservation of all nitrogenous material. There is a gain in weight of 0.7 kg.

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we are inclined toward the belief that this emergency *reserve store* is tucked away in the liver and perhaps other tissues as some "intermediate" substance—not as small aggregates like polypeptides but as large aggregates approximating proteins but not fixed tissue proteins. Probably such reserve store of protein building material can be used for other purposes than plasma protein regeneration—for example tissue or organ repair or red cell and hemoglobin regeneration.

Why should *iron feeding* stimulate plasma protein production—an excess output (Table 2) of 14.4 gm. above basal levels? We cannot say that iron enters into the reaction as is the case with its effect on the production of new hemoglobin. It is not known that iron modifies absorption from the intestinal tract; in fact iron is not readily absorbed even when in anemia due to blood loss there is an urgent need for it in internal metabolism.

The surplus plasma protein due to iron feeding probably comes from the basal food intake, as during fasting periods in the depleted dog the body proteins cannot contribute appreciably to the formation of new plasma protein. Let us argue that iron modifies internal metabolism so that new hemoglobin can be formed and further that as a result of this modified internal metabolism more materials emerge which can be built into new plasma protein. This is of the nature of a catalytic reaction. Fasting experiments with iron feeding and plasma depletion should give evidence bearing on this point.

SUMMARY

When blood plasma proteins are depleted by bleeding, with return of washed red cells (plasmapheresis) it is possible to bring the dog to a steady state of low plasma protein and uniform plasma protein production on a basal diet. Such dogs are excellent test subjects by which the potency of various diet factors for plasma protein regeneration can be measured.

To regenerate plasma proteins in any significant amount the depleted dog requires food protein. Some proteins are very potent for new plasma protein production and others are utilized poorly.

Beef serum is very potent and its proteins (2.6 gm.) will produce 1 gm. of new plasma protein in the depleted dog—a potency ratio of 2.6.

Kidney protein stands at the bottom of our list and the dog needs 21 gm. of kidney protein to regenerate 1 gm. of plasma protein—a potency ratio of 21.0.

Some grain proteins approximate the potency of beef serum and may show potency ratios of 2.7 to 4.6. Some of these grain proteins appear to favor the production of globulin more than albumin in the plasma.

Skeletal muscle, gizzard (smooth muscle), lactalbumin and egg white fall into a favorable group with a potency ratio of 5.3 to 6.0.

Whole liver, liver fractions, casein, and beef heart are a little less potent and present potency ratios of 6.5 to 8.0. Many of these food substances favor the production of albumin more than globulin.

Pancreas and salmon muscle show less favorable potency ratios of 19.0 and 15.0 respectively.

Fasting periods indicate that these depleted dogs can produce little if any new plasma protein.

Iron feeding in some unexplained manner will influence body metabolism so that an excess of plasma protein will be produced.

These observations have a bearing on clinical conditions associated with hypoproteinemia and give suggestions for diet aid or control in some of these abnormal states. The make-up of the diet is obviously of great interest and it is possible that protein combinations may be more potent than a single protein or that food potency ratios may differ in health and disease.

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DOG PLASMA PROTEIN GIVEN BY VEIN UTILIZED IN BODY METABOLISM OF DOG

HORSE PLASMA AND DOG HEMOGLOBIN NOT SIMILARLY UTILIZED

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Experiments given in this paper support the thesis (4) that dog plasma proteins when given intravenously to dogs are readily utilized in the body with the result that a positive nitrogen balance can be maintained during fasting periods. We knew that the anemic dog (9) promptly utilizes goose or sheep hemoglobin given intravenously to build abundant new hemoglobin and red cells. We anticipated therefore that the fasting dog would utilize *foreign plasma* much as it does dog plasma. This the body does not choose to do. All of the horse plasma protein given intravenously to the fasting dog is probably broken down and thrown away in the urine.

Hemoglobin is constantly being broken down in the circulation and in the presence of anemia the dog utilizes the globin fraction to make new hemoglobin (5). We suspected that when anemia was not present and when the body needed protein (fasting) there might be a demonstrable utilization of the globin fraction to spare or replace body protein. In this we were disappointed and we observe below in the fasting dogs that the bulk of the introduced hemoglobin is broken down and thrown away as extra nitrogen in the urine. There is evidence that some of the introduced hemoglobin is retained to form new hemoglobin for red cells.

* National Research Council Fellow.

Methods

Method and procedures have been described in an earlier paper (3). Dogs were normal healthy animals unless otherwise noted. All food was withheld for 3 days to allow nitrogen elimination to reach a fasting level. The dog was then catheterized and placed in a clean metabolism cage. Water was available in the cages at all times. When each experiment was terminated with catheterization the dogs were placed on the kennel diet and were not used again until they had regained their former weight, usually periods of 6-8 weeks intervening.

During the periods of dog plasma injection large healthy donors were bled 500 cc. into a flask using as an anticoagulant 22 mg. of *heparin* per 100 cc. of blood, dissolved in 5 cc. of Locke's solution. The blood was centrifugalized for 35 minutes at 3000 R.P.M. in 100 cc. centrifuge tubes, the plasma drawn off with suction, measured, warmed in a warm water bath to slightly above body temperature, and injected into the jugular vein or peritoneal cavity as indicated in each experiment. The total daily dosage was given in two injections 6-8 hours apart. Approximately 15 minutes were required for the plasma to run into the vein from a gravity bottle. A small sample was saved for analysis of total protein, albumin, and globulin. The metabolism cage was kept in the laboratory under close observation throughout the day and urine voided was put aside at once in a separate bottle containing sulfuric acid to prevent loss of nitrogen as ammonia. The urine output per period was diluted to a known volume (usually 500 cc.) and analyzed for total nitrogen and protein. A modification of the gravimetric method of Folin and Denis was used to determine urinary protein excretion.

To furnish horse plasma for the stated injections a normal horse was bled 500 cc. from the jugular vein into a flask, using as an anticoagulant 22 mg. of *heparin* per 100 cc. of blood. The plasma was analyzed for total protein, albumin, and globulin.

The dog hemoglobin for injection was prepared as described for other experiments (7). Healthy donors were bled 150 cc. into a flask containing 1.5 cc. of saturated sodium citrate. The cells were separated by centrifugalization and washed twice with normal saline. They were then laked with approximately twice their volume of distilled water by moderately severe shaking for at least 15 minutes. Following this the sediment was thrown down by high speed centrifugalization for at least 30 minutes and the clear supernatant fluid was filtered through several layers of cotton gauze. The filtrate was then ready for injection into the vein or peritoneal cavity. Hemoglobin was always used within 8 hours after preparation. All hemoglobin solutions were standardized by the acid hematin method (8). The urine obtained after hemoglobin injection was examined spectroscopically for traces of hemoglobin as described elsewhere (6). Clean but not sterile technique was used for the preparation of the fresh serum and hemoglobin.

Feces were collected promptly after defecation, preserved in sulfuric acid, dried over steam, pulverized, and aliquot portions analyzed for N. Usually the

total feces was handled as a unit and the daily average for the total period indicated in the tables.

EXPERIMENTAL OBSERVATIONS

To obviate some of the slight systemic reactions observed in the earlier experiments (4) which were attributed to sodium citrate we used *heparin* to prevent coagulation in collection of dog and horse plasma. This heparinized plasma can be given either intravenously or intraperitoneally with practically no reaction. In some animals as noted in their clinical histories there was vomiting. This vomitus contained little but mucus and only traces of nitrogen so that it was discarded—rarely did it contaminate the urine and even then it introduced no significant error.

Table 21 shows three separate experiments upon the same dog. This Dog 32-131 had been used in other metabolism experiments and in a previously published paper (4) is recorded a control experiment giving the dog sugar alone. This experiment serves as a base line control for those given in Table 21.

The first experiment (Table 21) is almost perfect. The heparinized plasma caused no clinical disturbance and the dog was normal in all respects. There was no contamination of the urine. In all these experiments the injected plasma was analyzed for protein content so that the figures given for plasma protein injection are accurate and not based on a 6 per cent estimated value as in the earlier report (4). These actual values were determined as 6.75 per cent for total protein. The dog received 160 gm. plasma protein by vein and there was no escape by way of the urine. At the end of the last control period the circulating plasma protein amounted to 55 gm. which is probably 30 gm. above a control on sugar alone. This accounts for about 20 per cent of the injected protein but leaves us 130 gm. retained in or used within the body. The urinary nitrogen is not increased by the plasma injections and the output of urinary N and intake of plasma N practically balance. The evidence seems conclusive that 130 gm. of plasma protein are utilized in the body economy. If this material was stored passively and then released we should expect a subsequent rise in the urinary N in the sugar after period.

During this same experiment there is no change in the A/G ratio although the plasma proteins rise to very high levels (9 per cent). In spite of this increase in plasma proteins there is but little change in

blood plasma volume and no spill over of protein in the urine. The red cell hematocrit falls steadily in proportion to the amount of bleeding which was considerable due to blood volume and other analyses and is reflected in the column "Protein removed from blood as N," Table 21.

TABLE 21

Plasma Protein and Hemoglobin Contrasted—by Vein and Intraperitoneally
Dog 32-131.

Experimental periods	Days	Injected N, daily average	Urinary N, daily average	Negative N balance, daily average	Protein removed from blood as N, daily average	Fecal N, daily average	Circulating plasma protein period end	Weight at period end
First Dog plasma by vein—sugar by stomach tube								
Fore period—sugar.....	5	0	2.07	2.35	0.182	0.1	39.48	15.3
Plasma protein 80.5 gm.....	7	1.84	1.83	0.32	0.230	0.1	48.5	14.3
Plasma protein 79.4 gm.....	7	1.814	1.99	0.61	0.337	0.1	60.79	13.8
After period—sugar.....	5	0	1.294	1.64	0.242	0.1	55.1	13.0
Second Dog plasma intraperitoneally—sugar and vegetable oil by stomach tube								
Fore period—sugar.....	5	0	2.50	3.36	0.083	0.78	50.45	15.8
Plasma protein 180.2 gm.....	8	3.603	2.32	+0.34	0.172	0.78	82.72	15.4
After period—sugar.....	5	0	3.21	4.13	0.141	0.78	52.00	14.4
Third Dog hemoglobin by vein—sugar by stomach tube								
Fore period—sugar.....	5	0	1.744	2.21	0.054	0.41	41.82	14.2
Hemoglobin 30.2 gm.....	7	0.690	2.181	1.96	0.060	0.41	35.48	14.0
Hemoglobin 30.8 gm.....	7	0.70	2.146	1.92	0.068	0.41	38.15	13.2
After period—sugar.....	5	0	1.154	1.67	0.108	0.41	34.84	12.6

Intraperitoneal injections of dog plasma (Dog 32-131, second period) give results in general in accord with the intravenous experiment on the same dog. Larger amounts of plasma can be given (180 gm. in 8 days) and there is no escape by way of the urine. The injected N (3.6 gm. daily) much exceeds the urinary N (2.3 gm. daily) so that there is a positive N balance.

Vomiting developed during the last part of the plasma injection period and continued during the after period of sugar feeding. This may have been due in part to the stomach tube feeding of oil with the sugar causing nausea but probably in part to slight peritoneal irritation due to repeated plasma injections. As a result of the loss of

TABLE 21-a

Plasma Protein, Urinary Protein, Plasma and Red Cell Volume
Dog 32-131.

Experimental periods	Days	Blood plasma Average concentration			A/G ratio	Urinary N, as protein N, daily average	N.P.N. plasma	Plasma volume period end	R.H.C. hematocrit period end
		Total protein	Albumin	Globulin					
First Dog plasma by vein—sugar by stomach tube									
		per cent	per cent	per cent		gm.	mg. per cent	cc.	per cent
Fore period—sugar.....	5	5.64	3.02	2.62	1.15	0	13	696	46.0
Plasma—dog.....	7	8.11	4.24	3.87	1.10	0		714	31.6
Plasma—dog.....	7	8.19	4.26	3.93	1.08	0	20	781	26.5
After period—sugar.....	5	7.59	4.16	3.35	1.21	0		652	27.8
Second Dog plasma intraperitoneally—sugar and vegetable oil by stomach tube									
Fore period—sugar.....	5	6.37	3.21	3.16	1.02	0	20	792	46.0
Plasma—dog.....	8	9.60	5.54	4.06	1.36	0		1043	34.1
After period—sugar.....	5	8.15	4.38	3.77	1.16	0	20	638	42.1
Third Dog hemoglobin by vein—sugar by stomach tube									
Fore period—sugar.....	5	6.56	3.61	2.95	1.22	0		648	46.0
Hemoglobin.....	7	5.40	2.65	2.75	0.96	0.011		666	49.4
Hemoglobin.....	7	6.28	3.02	3.26	0.93	0.009	19	549	51.6
After period—sugar.....	5	6.25	3.63	2.62	1.39	0.004	17	566	45.5

sugar there is a rise in the urinary N but we must admit that peritoneal irritation might be responsible for a little of this urinary N excess in the after period.

The plasma protein retention following intraperitoneal introduction seems to be the same as with intravenous injection. The rise in circulating plasma proteins is extreme but the rise in plasma volume is

not in proportion although present (Table 21-a, second period). The A/G ratio changes but little.

Hemoglobin given by vein (Table 21, third period) in the same dog (32-131) seems to escape in the form of urinary N except for some which may be utilized to form new hemoglobin for new red cells. In giving hemoglobin by vein in these experiments it is necessary to keep below the renal threshold (5) for hemoglobin in the dog. In fact on only a few occasions were traces of hemoglobin noted in the urine and the protein N escape in the urine is trivial. Only 30 gm. of hemoglobin could be given intravenously each week and still keep below the renal threshold and this amounts to 0.7 gm. N per day. As a result of this injection the urinary N rises 0.5–0.6 gm. per day above the expected levels—indicating that there is practically no N retention—and the usual negative urinary N balance continued. Meanwhile the red cell volume and hematocrit do not fall in contrast to an experiment on the same dog with plasma injection (first period) where the red cell hematocrit fell from 46–28 per cent. We may argue therefore that some of the injected hemoglobin is used to make new hemoglobin for red cells and the rest is discarded to account for the excess urinary N. Certainly the reaction to *hemoglobin* is very different from that to *plasma* when either is given intravenously to a protein fasting dog.

Clinical History, Dog 32-131.—A young mongrel hound. See Tables 21 and 21-a. First experiment, Sept. 29. Weight 16.7 kg. Plasma protein 5.58 per cent; albumin 2.73 per cent; globulin 2.58 per cent; plasma volume 724 cc.; blood volume 1350 cc.; red cell hematocrit 46.0 per cent. Sept. 29–Oct. 1, all food withheld, weight fell to 15.9 kg. Oct. 2, 50 gm. glucose and 300 cc. water daily by stomach tube. 10 gm. kaolin added on alternate days. Catheterization to start metabolism experiment. Oct. 7, dog plasma injection begun, intravenously. Catheterization. Oct. 9, more or less generalized urticaria $\frac{1}{2}$ hour after injection. 1 cc. of 1:1000 adrenalin was given subcutaneously and by the next day the reaction had cleared and the rest of the experiment was without event. Oct. 14, catheterization. Oct. 17, no reaction using plasma from same donor as on Oct. 9. Oct. 20, last plasma injection. Oct. 21, catheterization. Oct. 26, final catheterization. No fecal contamination of urine during entire period. Dog put on kennel diet.

Second experiment, Tables 21 and 21-a. Apr. 14, weight 17.5 kg. Plasma protein 6.50 per cent; albumin 3.48 per cent; globulin 3.02 per cent; N. P. N. 25 mg.; plasma volume 966 cc.; blood volume 1756 cc.; red cell hematocrit 45 per cent.

Apr. 15-17, all food withheld. Apr. 18, daily 100 gm. glucose plus 100 cc. Wesson oil (vegetable oil) plus 10 gm. kaolin, plus 350 cc. water, by stomach tube. Catheterization to start metabolism experiment. Apr. 23, catheterization. Dog plasma injection intraperitoneally begun. May 1, catheterization. Last plasma injection. Vomiting occurred throughout the period of injection, and at times there was contamination of the urine by vomitus. May 6, catheterization. Dog placed on kennel diet.

Third experiment, Tables 21 and 21-a. Nov. 24, weight 16.2 kg. Plasma protein 6.16 per cent; albumin 3.83 per cent; globulin 2.33 per cent; N.P.N. 17 mg.; plasma volume 763 cc.; blood volume 1384 cc.; red cell hematocrit 45.2 per cent. Nov. 24-26, all food withheld. Nov. 27, weight 15.2 kg. Plasma protein 7.04 per cent; plasma volume 627 cc.; blood volume 1160 cc.; red cell hematocrit 46.2 per cent. Daily, 60 gm. dextrose plus 10 gm. kaolin plus 300 cc. water by stomach tube. Catheterization. Dec. 1, catheterization to start metabolism experiment. Hemoglobin injections started, intravenously. Dec. 6, hemoglobin in urine gross +; spectroscopically +. This is the only occasion upon which the dog showed hemoglobinuria either by gross or spectroscopic examination. Dec. 8, catheterization. Dec. 14, last hemoglobin injection. Dec. 15, catheterization. Dec. 19, final catheterization. Dog returned to kennel.

Table 22 gives four experiments on four different dogs. The first experiment (Dog 33-324, Table 22) is much like that described above (Dog 32-131, Table 21). Heparinized dog plasma was given in large amounts and sugar was given in larger doses although to a smaller dog. During the 8 days of plasma injection the dog actually gained 0.2 kg. of body weight and the urinary N is not increased by the plasma injection—there is a small positive balance between the intake N and the urinary N. In the after period we see the urinary N at the level expected from sugar feeding alone and there is no escape of any stored N.

The plasma proteins (Table 22-a, Dog 33-324) show conspicuous increases due to the plasma injection but the A/G ratio is unchanged. There is no escape of protein in the urine. The usual fall in the red cell hematocrit is observed due to frequent sampling of blood.

Clinical History, Dog 33-324.—See Tables 22 and 22-a, first experiment. May 18, plasma protein 5.56 per cent; albumin 3.48 per cent; globulin 2.08 per cent; N.P.N. 11 mg. May 25-28, all food withheld. May 28, weight 9.7 kg. Daily, 60 gm. glucose, plus 10 gm. kaolin in 300 cc. water, by stomach tube. Catheterization. June 2, dog plasma injection intravenously started. June 4, following the injection the dog developed a marked urticaria and vomited a considerable

portion of its sugar. Vomiting occurred daily thereafter throughout the period of injection. The urine was not contaminated by vomitus however. June 9, last plasma injection. June 10, catheterization. June 15, final catheterization. Dog put on kennel diet.

TABLE 22
Foreign Plasma Protein and Dog Hemoglobin Not Conserved by the Protein Fasting Dog

Experimental periods	Days	Injected N, daily average	Urinary N, daily average	Negative N balance, daily average	Protein removed from blood as N, daily average	Fecal N, daily average	Circulating plasma protein period end	Weight at period end
Dog 33-324 Dog plasma by vein—sugar by stomach tube								
Fore period—sugar.....	5	0	1.920	2.07	0.047	0.1	18.04	9.0
Plasma protein 98.4 gm.....	8	1.968	1.920	0.20	0.092	0.16	41.29	9.2
After period—sugar.....	5	0	1.440	1.66	0.113	0.11	27.48	8.3
Dog 33-136 Horse plasma by vein—sugar by stomach tube								
Fore period—sugar.....	5	0	3.388	4.02	0.110	0.52	33.86	14.7
Plasma protein 103.7 gm.....	7	2.371	4.169	2.47	0.150	0.52	44.87	13.6
Plasma protein 40.8 gm.....	3	2.183	7.553	5.91	0.017	0.52	56.92	13.6
After period—sugar.....	4	0	7.748	8.54	0.272	0.52	47.17	13.6
Dog 31-138 Horse plasma intraperitoneally—sugar by stomach tube								
Fore period—sugar.....	5	0	3.126	4.10	0.101	0.87	50.76	22.6
Plasma protein 105.0 gm.....	7	2.401	3.619	2.20	0.112	0.87	54.37	21.7
After period—sugar.....	4	0	3.297	4.31	0.140	0.87	59.59	21.3
After period—sugar.....	4	0	3.900	4.90	0.127	0.87	54.15	20.1
Dog 33-178 Dog hemoglobin intraperitoneally—sugar by stomach tube								
Fore period—sugar.....	5	0	2.094	2.76	0.066	0.60	29.61	15.6
Hemoglobin 80.0 gm.....	7	1.83	3.091	1.92	0.061	0.60	37.66	14.8
Hemoglobin 44.8 gm.....	4	1.79	3.505	2.378	0.063	0.60	43.20	14.3
After period—sugar.....	4	0	2.225	3.01	0.185	0.60	39.59	14.0

The second experiment (Dog 33-136, Table 22) shows the result of giving *horse plasma* intravenously in amounts corresponding to the dog plasma experiments. The injections were continued for 10 days

at which time evidences of "serum hypersensitivity" developed together with albuminuria and the dog was clinically quite sick. The N.P.N. of the blood rose (Table 22-a). Several days later the dog was

TABLE 22-a
Plasma Protein, Urinary Protein, Plasma and Red Cell Volume

Experimental periods	Days	Blood plasma Average concentration			A/G ratio	Urinary N as protein N ₂ daily average	N.P.N plasma	Plasma volume period end	R.B.C. hematocrit period end
		Total protein	Albumin	Globulin					
Dog 33-324 Dog plasma by vein—sugar by stomach tube									
		per cent	per cent	per cent		gm.	mg. per cent	cc.	per cent
Fore period—sugar.....	5	4.89	2.97	1.92	1.6	0	18		53.2
Plasma—dog.....	8	7.91	4.75	3.16	1.5	0		522	26.1
After period—sugar.....	5	7.25	4.34	2.91	1.5	0	18	379	36.1
Dog 33-136 Horse plasma by vein—sugar by stomach tube									
Fore period—sugar.....	5	4.87	2.84	2.03	1.40	0.005	16	525	47.3
Plasma—horse.....	7	8.13	3.81	4.32	0.88	0.061	23	904	37.4
Plasma—horse.....	3	6.20	2.72	3.48	1.28	1.660	48	746	33.0
After period—sugar.....	4	5.66	2.43	3.23	1.33	0.927	49	845	25.5
Dog 31-138 Horse plasma intraperitoneally—sugar by stomach tube									
Fore period—sugar.....	5	5.97				0	15	791	53.0
Plasma—horse.....	7	6.83	3.48	3.35	1.04	0.004	21	947	46.0
After period—sugar.....	4	6.11	3.33	2.78	1.19	0.010	23	896	44.0
After period—sugar.....	4	5.95	3.12	2.83	1.10	0.012	23	900	46.0
Dog 33-178 Dog hemoglobin intraperitoneally—sugar by stomach tube									
Fore period—sugar.....	5	5.69	3.23	2.46	1.31	0	17	570	54.0
Hemoglobin.....	7	6.46	2.48	3.98	0.62	0.002	18	671	53.6
Hemoglobin.....	4	6.60	2.34	4.26	0.55	0.003	15	652	50.0
After period—sugar.....	4	5.38	3.20	2.18	1.47	0.002	16	670	51.0

killed with ether (clinical history below). A chronic nephritis found at autopsy explains in part the high urinary escape of protein.

Horse plasma intravenously (Dog 33-136, Table 22) reacts entirely differently from dog plasma. The urinary N rises to high levels fol-

lowing these injections and in the after period continues at even higher levels. In fact there is an escape of more N in the urine above control levels than was introduced, to be explained no doubt by the body reaction to a foreign protein with the associated clinical symptoms of intoxication. In spite of this loss of urinary N the circulating plasma contains an excess of protein during and after the horse plasma injection periods. The A/G ratio is disturbed in the 1st week (Table 22-a) and the N.P.N. rises to 3 times normal. The surplus of circulating protein may be thought to be a mixture of horse and dog plasma protein and this in fact may be the case but during the after period this excess might be due to a contribution from the reserve store which we know can furnish much new plasma protein. The reaction to foreign serum complicates this experiment and makes it unsatisfactory.

The rise in plasma volume (Dog 33-136, Table 22-a) is of some interest and may be wholly due to the excess of plasma protein but some may choose to explain this apparent rise in plasma volume as due to increased permeability of the capillaries sufficient to permit escape of the blood volume dye thus giving high readings for blood volume

The red cell hematocrit shows the usual fall due to repeated blood samplings although some of this fall may be explained by the increase in blood volume if this is real and not due to dye escape.

Clinical History, Dog 33-136.—See Tables 22 and 22-a, second experiment. Jan. 5, weight 16.6 kg. Plasma protein 5.07 per cent; albumin 3.25 per cent; globulin 1.82 per cent; plasma volume 858 cc.; blood volume 1619 cc.; red cell hematocrit 47 per cent. Jan. 5-7, all food withheld. Jan. 8, plasma protein 5.21 per cent; albumin 3.38 per cent; globulin 1.83 per cent; N.P.N. 22 mg.; plasma volume 775 cc.; blood volume 1490 cc.; red cell hematocrit 48.1 per cent. Daily, 60 gm. glucose plus 300 cc. water, by stomach tube. Catheterization. Jan. 13, catheterization. First injection of horse plasma, intravenously. Jan. 22, last plasma injection. Dog quite lethargic. Icteric. Areas of hyperemia are prominent over the body surface. Jan. 23, catheterization. Dog more active. Jan. 26, catheterization. Dog returned to kennel diet. Feb. 3, plasma protein 3.17 per cent; albumin 1.43 per cent; globulin 1.64 per cent; N.P.N. 56 mg. Dog has become progressively more listless since returned to kennel. Now comatose. Shows marked edema. Dog killed and autopsied. Autopsy findings: Necrotizing bronchopneumonia. Pulmonary edema. Acute splenic tumor. Acute lymphadenitis. Chronic nephritis. Cloudy swelling of kidneys. Ulcerations of skin. Ascites. Marked edema of lower extremities.

Horse plasma given for 7 days *intra-peritoneally* is a satisfactory experiment (Dog 31-138, Table 22) and again gives no positive evidence that the body can utilize these foreign proteins as it does dog plasma protein. Horse plasma protein (105 gm.) is equivalent to 16.8 gm. of N and if this dog had been on sugar alone we believe its final after period would have shown a urinary daily output of 2.5 gm. N. If we calculate the excess due to the plasma injections we find 120 gm. N or about 75 per cent of the injected N. This dog on sugar alone would show a shrinkage of circulating proteins probably to 30 gm. or less, a level 25 gm. below the end period in this experiment. This surplus plus the protein escaping in the urine will account for all of the injected horse plasma protein. This assumes that horse plasma protein remains in the circulation. It would be of interest to expand the after period to observe whether for a time an excess of urinary N would be observed while the blood plasma proteins are returning to normal. Certainly it would be a rash claim that the foreign protein is utilized within the body metabolism.

Clinical History, Dog 31-138.—See Tables 22 and 22-a, third experiment. Feb. 26, weight 23.8 kg. Plasma protein 6.12 per cent; albumin 3.80 per cent; globulin 2.32 per cent; N.P.N. 20 mg.; plasma volume 913 cc.; blood volume 2077 cc.; red cell hematocrit 56 per cent. Feb. 26-28, all food withheld. Mar. 1, weight 23.1 kg.; plasma protein 6.04 per cent; albumin 4.75 per cent; globulin 1.29 per cent; N.P.N. 28 mg.; plasma volume 901 cc.; blood volume 1936 cc.; red cell hematocrit 54 per cent; daily 80 gm. glucose by stomach tube. Catheterization. Mar. 6, catheterization. Horse plasma injection, intraperitoneally, begun. Marked urticarial reaction 30 min. after initial injection. Vomited 3 hours after injection. Mar. 11, retched after injection, no vomitus. Urine contaminated by feces. Mar. 12, last plasma injection. Mar. 13, catheterization. Mar. 17, catheterization. Mar. 21, final catheterization. Dog normal and returned to kennel.

Dog hemoglobin given intra-peritoneally (Dog 33-178, Table 22) proves to be a very interesting experiment. About 125 gm. of dog hemoglobin (or a total of about 20 gm. of N) was given into the peritoneum during 11 days. During the two periods of injection and the after period an excess of urinary N amounting to about 17 gm. was found. This accounts for practically all the injected hemoglobin. We note only a trivial fall in the red cell hematocrit as contrasted with other like experiments without hemoglobin injections. We may argue

therefore that a little of the injected hemoglobin was utilized to keep up the high level of the red cell hematocrit. This would seem to preclude any possibility that the body could use the *globin* from hemoglobin as it does injected dog plasma proteins in other experiments.

One significant observation deserves comment (Table 22-a, Dog 33-178). The A/G ratio changes conspicuously and falls to 0.62 and 0.55 while the globulin fraction shows a great increase. It is suggested that this may be due to the *globin* from the hemoglobin after the pigment radicle has been split off. It would appear in the globulin fraction by the method used. The A/G ratio promptly comes back to normal when the hemoglobin injections are discontinued as presumably the globin was broken down and discarded as excess urinary N. During this period the plasma showed bile pigmentation but not any red color. The same type of change is noted when the hemoglobin is given by vein (Table 21-a, third period) but it is not as conspicuous.

This apparent increase in globulin and total plasma proteins may be due to the *globin* from injected hemoglobin, explaining the high values for total circulating plasma protein.

Clinical History, Dog 33-178.—See Tables 22 and 22-a, fourth experiment. Feb. 5, weight 17.0 kg. Plasma protein 5.61 per cent; albumin 3.82 per cent; globulin 1.79 per cent; N.P.N. 21 mg. Feb. 5-7, all food withheld. Feb. 8, weight 15.9 kg., plasma protein 5.35 per cent; albumin 2.84 per cent; globulin 2.51 per cent; plasma volume 503 cc.; blood volume 1061 cc.; red cell hematocrit 52.0 per cent. Daily, 70 gm. dextrose plus 10 gm. kaolin, plus 300 cc. water, by stomach tube. Catheterization. Feb. 13, intraperitoneal hemoglobin injection started. Catheterization to start metabolism experiment. Feb. 14, hemoglobin in urine, gross ++; spectroscopically ++. Feb. 20, hemoglobin in urine, gross ?; spectroscopically +. Catheterization. Vomited immediately after injection. Appeared lethargic for following 18-20 hours. Feb. 23, hemoglobin in urine gross 0; spectroscopically +. Last hemoglobin injection. Feb. 24, catheterization. Feb. 28, catheterization. Dog returned to kennel.

DISCUSSION

When we found that new hemoglobin in large amounts could be produced in the anemic dog while fasting (1) and recalled the observation (2) that much new liver protein could be regenerated during protein fasting (chloroform poisoning and liver repair during sugar periods), when we observed that from a protein reserve plasma

proteins could be produced in large amounts above the basal output we used the term "dynamic equilibrium" to indicate an ebb and flow between various organ, plasma, and body proteins. We were tempted to picture these *protein reserves* as water spiders skittering about on the mill pond of body metabolism activated by the stimulus of the moment. It would appear that there are patterns for the actions of these water spiders and the pattern for plasma protein movement and construction is more limited than the pattern for hemoglobin protein.

To produce abundant plasma protein the dog must have suitable diet food factors or draw upon the reserve protein store and when this reserve is depleted so far as we can see the dog is almost wholly dependent upon food protein to make new plasma protein. There is evidence (Table 4, Paper I) that when the protein reserve is exhausted, during fasting periods the dog can produce little if any new plasma protein. This has a bearing upon the *nature of this reserve store* of protein building material for plasma protein. It would seem that the reserve store was not in the form of fixed and mature organ protein otherwise we would expect some plasma protein regeneration during fasting periods when body protein is being broken down. It is possible that this reserve store is in the form of an immature or pro protein material of large molecular form stored in various organs, for example the liver. These substances may be designated as "intermediates."

The possibilities are much more varied and our pattern of behavior for *hemoglobin* much more elaborate as the anemic dog can produce new hemoglobin not only from diet factors and from a reserve protein store but by conservation of protein waste products normally present during any fasting period (1). Foreign hemoglobins (sheep and goose) are promptly picked up and recast to form new dog hemoglobin in anemia, and hemoglobin destroyed in the anemic dog is promptly turned over into new hemoglobin and red cells.

There was a real reason why we wished to know whether the dog could utilize the globin from hemoglobin during periods of emergency. In the plasmapheresis experiments we return an excess of washed red cells because many of these introduced cells rapidly go to pieces in the circulation. The washing and other trauma presumably injure the red cells and shorten their life in the circulation. Could this excess

therefore that a little of the injected hemoglobin was utilized to keep up the high level of the red cell hematocrit. This would seem to preclude any possibility that the body could use the *globin* from hemoglobin as it does injected dog plasma proteins in other experiments.

One significant observation deserves comment (Table 22-a, Dog 33-178). The A/G ratio changes conspicuously and falls to 0.62 and 0.55 while the globulin fraction shows a great increase. It is suggested that this may be due to the *globin* from the hemoglobin after the pigment radicle has been split off. It would appear in the globulin fraction by the method used. The A/G ratio promptly comes back to normal when the hemoglobin injections are discontinued as presumably the globin was broken down and discarded as excess urinary N. During this period the plasma showed bile pigmentation but not any red color. The same type of change is noted when the hemoglobin is given by vein (Table 21-a, third period) but it is not as conspicuous.

This apparent increase in globulin and total plasma proteins may be due to the *globin* from injected hemoglobin, explaining the high values for total circulating plasma protein.

Clinical History, Dog 33-178.—See Tables 22 and 22-a, fourth experiment. Feb. 5, weight 17.0 kg. Plasma protein 5.61 per cent; albumin 3.82 per cent; globulin 1.79 per cent; N.P.N. 21 mg. Feb. 5-7, all food withheld. Feb. 8, weight 15.9 kg., plasma protein 5.35 per cent; albumin 2.84 per cent; globulin 2.51 per cent; plasma volume 503 cc.; blood volume 1061 cc.; red cell hematocrit 52.0 per cent. Daily, 70 gm. dextrose plus 10 gm. kaolin, plus 300 cc. water, by stomach tube. Catheterization. Feb. 13, intraperitoneal hemoglobin injection started. Catheterization to start metabolism experiment. Feb. 14, hemoglobin in urine, gross ++; spectroscopically ++. Feb. 20, hemoglobin in urine, gross ?; spectroscopically +. Catheterization. Vomited immediately after injection. Appeared lethargic for following 18-20 hours. Feb. 23, hemoglobin in urine gross 0; spectroscopically +. Last hemoglobin injection. Feb. 24, catheterization. Feb. 28, catheterization. Dog returned to kennel.

DISCUSSION

When we found that new hemoglobin in large amounts could be produced in the anemic dog while fasting (1) and recalled the observation (2) that much new liver protein could be regenerated during protein fasting (chloroform poisoning and liver repair during sugar periods), when we observed that from a protein reserve plasma

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There was a real reason why we wished to know whether the dog could utilize the globin from hemoglobin during periods of emergency. In the plasmapheresis experiments we return an excess of washed red cells because many of these introduced cells rapidly go to pieces in the circulation. The washing and other trauma presumably injure the red cells and shorten their life in the circulation. Could this excess

hemoglobin be utilized to make plasma protein or other body proteins? The evidence is against this possibility as given above.

Evidence that the dog cannot utilize foreign plasma protein and cannot utilize the globin from hemoglobin except to form new hemoglobin for red cells has been given. This evidence by contrast actually strengthens the case for the ready utilization of dog plasma protein when given parenterally during protein fasting. We should think of the plasma proteins as being continuously replenished from the food factors as they are called on for various protein needs within the body.

SUMMARY

Foreign plasma protein (horse) introduced parenterally into the protein fasting dog is not utilized in the body economy. Its fate appears to be disintegration and elimination as excess urinary nitrogen. This is totally different from the fate of dog plasma protein under similar conditions.

Dog hemoglobin given parenterally to the protein fasting dog is not utilized as is dog plasma protein to keep the animal in nitrogen equilibrium but the globin is largely broken down and discarded as excess urinary nitrogen. A small part of the injected hemoglobin is probably utilized to maintain the red cell concentration in the blood at high levels.

Dog plasma given parenterally in a protein fasting dog will maintain the dog in nitrogen equilibrium and there is no surplus nitrogen elimination in the after periods. It is apparent that the introduced plasma protein is utilized efficiently in body metabolism to replace or repair tissue protein. It is suggested that although this is an emergency reaction the same reactions may go on in normal internal metabolism. The observation that *foreign* plasma and dog hemoglobin cannot be utilized when given parenterally actually strengthens this last argument for a normal contribution from plasma proteins to body proteins.

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NUTRITIONAL EDEMA IN THE DOG

I. DEVELOPMENT OF HYPOPROTEINEMIA ON A DIET DEFICIENT IN PROTEIN

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Two years ago in a paper from this laboratory (1) some observations were presented on the development of nutritional edema in the dog. The observations were based on experiments with seven animals which developed hypoproteinemia and edema after subsisting for variable times on a diet low in protein. Since the time of that publication the experimentally induced edema has been utilized as a means of studying particular aspects of the edema problem (2) and a considerable array of additional data has been accumulated. In the light of this further experience it appears desirable to describe in more detail the events which have been observed. The present communication will deal with the behavior of the serum and tissue proteins during the experiments.

Methods

Animals.—25 dogs of mongrel breeds used in these experiments varied in weight from 13 to 22 kilos and averaged 17 kilos. Young adult dogs were selected by preference; their ages, not definitely known, were estimated to be between 1 and 3 years.

Diet.—The first eight dogs in the series were maintained either on a synthetic diet or a natural food diet, both of which have been described previously (1). For the remaining seventeen dogs the composition of the natural food diet was modified by removing the butter fat and supplying its nutritional equivalent with lard and cod liver oil. The composition of the modified diet follows:

Carrots.....	300
Rice.....	35
Lard.....	40

	gm.
Cod liver oil.....	10
Sugar.....	115
Salt mixture.....	5

The carrots and rice were cooked together for 20 minutes in a small amount of water; the lard, cod liver oil, sugar, and salt were added after cooking but while the mixture was still hot. Finally water was added until the mixture weighed 900 gm. This amount represented the quantity offered to each dog daily irrespective of its size. It furnished 1,200 calories as calculated from the tables of Atwater and Bryant (3) and by our own analysis contained 1.23 gm. nitrogen.

The salt mixture was similar to that employed by Gamble, Putnam, and McKhann (4) except that it contained sodium chloride and a larger amount of iron. Its composition follows:

	gm.
Calcium carbonate (CaCO_3).....	26.0
Magnesium carbonate, basic ($3\text{MgCO}_3 \cdot \text{Mg}(\text{OH})_2 \cdot 3\text{H}_2\text{O}$).....	6.1
Potassium chloride (KCl).....	22.4
Sodium chloride (NaCl).....	16.8
Sodium acid phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$).....	20.6
Sodium carbonate (Na_2CO_3).....	4.1
Ferric ammonium citrate ($\text{Fe}_2(\text{NH}_4)_2(\text{C}_6\text{H}_5\text{O}_7)_2 \cdot 3\text{H}_2\text{O}$).....	4.0

Additions to and subtractions from the salt mixture were made from time to time with some dogs and will be described as the occasion arises. During all experiments no restriction was placed on the intake of water.

Metabolism Observations.—During the experiments the dogs were kept in metabolism cages and their natural activity was therefore restricted. The type of cage permitted complete collection of stool and urine. Stools were transferred daily to a container kept at refrigeration temperature and were covered with sufficient distilled water to prevent desiccation. At the end of each period, usually 7 days, the total collection with added water was weighed, and then thoroughly stirred with a mechanical mixer for approximately $\frac{1}{2}$ hour. Weighed aliquots from the resulting homogeneous mass were used for analysis. Urine was collected in bottles containing a small amount of toluol and the daily volume carefully measured. The urines from each metabolism period were usually combined before analysis although in some instances the daily voidings were analyzed separately. At the end of each period the cages were washed thoroughly with approximately 2 liters of distilled water and the washings saved for analysis. The intake of the elements studied was calculated from the known composition of the diet and the weighed quantity of food consumed each day. Only distilled water was offered for drinking during periods of metabolism study.

Blood Samples.—Blood for analysis was obtained at intervals of from 1 to 3 weeks by puncture of the femoral artery. In some experiments the analyses were made on serum and in some on plasma in which coagulation had been prevented

by the addition of heparin, 1 mg. for each cc. of blood. The samples were not protected with oil against loss of CO_2 as it was not possible to demonstrate significant change in the blood protein when this precaution was taken.

Analytical Procedure.—Methods for the determination of nitrogen and of the serum or plasma proteins have been described previously (1). The micro Kjeldahl apparatus for distillation of ammonia in steam, which we have used, is similar to that described by Peters and Van Slyke (5) and by these authors is attributed to Goebel. A calculation of the accuracy, or better the reproducibility, of analytical results for protein obtained by this method has been made from data secured in duplicate determinations on 152 samples of serum.¹ The calculation for total protein expressed as grams per 100 cc. shows a probable error of measurement (PE_M) of 0.077 for a single determination and a PE_M of 0.054 for analyses which represent the average of duplicate determinations. All analyses in this investigation were performed in duplicate. The result means that the chances are even (50-50) that a given analytical figure will not differ by more than 0.054 gm. from the theoretical average of an infinity of determinations on the same sample. Furthermore, the chances are 993 in 1,000, *i.e.* practically certain, that the figure will not differ from the true average by more than four times this amount or 0.22 gm. The calculation in the case of albumin reveals a PE_M of 0.069 gm. per cent for a single determination and a PE_M of 0.050 gm. per cent for analyses which represent the average of duplicate determinations.

Blood Proteins of Normal Dogs

An examination of the data from these and other experiments discloses 57 analyses of the serum or plasma proteins in normal dogs. The average results with accompanying statistical data are presented in Table I. In general the figures for total protein agree with those presented by other authors although there is considerable discrepancy in the albumin and globulin fractions; that is, our series has yielded lower levels for albumin and higher levels for globulin. In 35 normal dogs Hurwitz and Whipple (6) found, by a microrefractometric method, concentrations of albumin between 3.5 and 6.0 gm. per cent and an average concentration of 4.6 gm. per cent. In this series the average albumin concentration was 3.3 gm. per cent and the individual findings were between 2.3 and 4.3 gm. per cent. Similarly Hurwitz and Whipple reported globulin variations between 0.4 and 2.8 gm. per cent and an average of 1.5 gm. per cent, whereas in our series

¹ The methods of statistical analysis employed in this paper were taken from: Garrett, H. E., *Statistics in psychology and education*, New York, Longmans, Green and Co., 1926.

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Cod liver oil.....	10
Sugar.....	115
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of 38 serum analyses the range was from 1.8 to 5.4 gm. per cent and the average value was 2.7 gm. per cent. The values reported in 1883 by Burckhardt (7), who used an antiquated and cumbersome method of analysis, digress even further in the direction of high levels for albumin and low levels for globulin. It is possible that difference in diet and previous illness may be factors in explaining the discrepancies. However, they probably result chiefly from difference in analytical procedure.

The statistical data in Table I show that globulin exhibits a wider range of normal variation than albumin. Indeed the variations en-

TABLE I
Proteins in Serum and Plasma of Normal Dogs

	Average per 100 cc.	Standard deviation of distribution	Standard error of average	Coefficient of variation
	Serum (38 dogs)			
	gm.			
Albumin.....	3.26	0.48	0.08	14.8
Globulin.....	2.72	0.76	0.12	27.8
Total protein.....	5.98	0.67	0.11	11.3
	Plasma (19 dogs)			
Albumin.....	3.38	0.38	0.09	11.2
Globulin.....	2.98	0.55	0.13	18.4
Total protein.....	6.36	0.71	0.16	11.2

countered in the globulin analyses are greater than those recorded for total protein. The fact suggests that albumin and globulin are not completely independent variables but that there is a certain tendency for low albumin to be associated with high globulin and *vice versa*. To evaluate the possible relationship we have determined the coefficient of correlation between albumin and globulin in the 38 analyses of serum and found it to be -0.49 .² The probable error of the coefficient

² A negative correlation coefficient between two variables indicates that the relation is inverse, that is, that a large degree of one is associated with a low degree of the other and *vice versa*.

is 0.08. This means that the chances are even that the true coefficient falls within the limits -0.49 ± 0.08 and that the chances are 9,999 in 10,000 that it has at least a negative value. It is therefore certain that a negative correlation does exist between the albumin and globulin of normal dogs but with the data available it is not possible to prove a high correlation.

The averages given in Table I also show that the globulin of plasma is slightly higher than the globulin of serum, the difference being 0.26 gm. per cent. The number of animals is not sufficient to prove that the difference is significant, although it is apparent that a real difference must exist from the circumstance that fibrinogen and serum globulin are included together when plasma is analyzed by the method used in this investigation. In subsequent portions of this paper in which the trend of the serum proteins will be pictured from average results in a group of animals, we have diminished the values for globulin and total protein by 0.3 gm. per cent when plasma rather than serum was analyzed. This means of adjusting plasma and serum analyses to a common basis is of course only approximate but because of the relatively small size of the fibrinogen fraction it cannot introduce significant errors. The analyses were about equally divided between serum and plasma.

Effect of Inadequate Dietary Protein on Serum Proteins

The protocols disclose 21 animals in which diet was the only significant factor in the production of hypoproteinemia for a period of 50 days. In several other experiments initial periods of plasmapheresis render the data unsuited for studying the uncomplicated effect of diet. With some of the 21 animals the effect could be followed for a longer time; with eight dogs through the 80th day and with three dogs for a hundred days or longer. Altogether 150 determinations of albumin, globulin, and total protein are available for analysis. The results for albumin and total protein are presented graphically in Chart 1. In order to construct a composite curve from the results, the serum proteins were calculated by interpolation for each dog at 10 day intervals throughout the period of observation. The average values at these intervals together with their standard deviations are presented in Chart 2. Both charts indicate the downward trend of albumin and

total protein with continuance of the diet. It is seen more clearly in Chart 2 that the decline is most rapid during the early days on the diet, that it becomes progressively slower during subsequent periods, and that terminally the rate of fall is again accelerated. The terminal rapid fall is seen better in the separate protocols and is somewhat obscured in the curves of averages because of its occurrence at different times in different animals.

Chart 2 also shows that average globulin concentration is almost unaffected by the diet. The terminal values are very slightly, and not

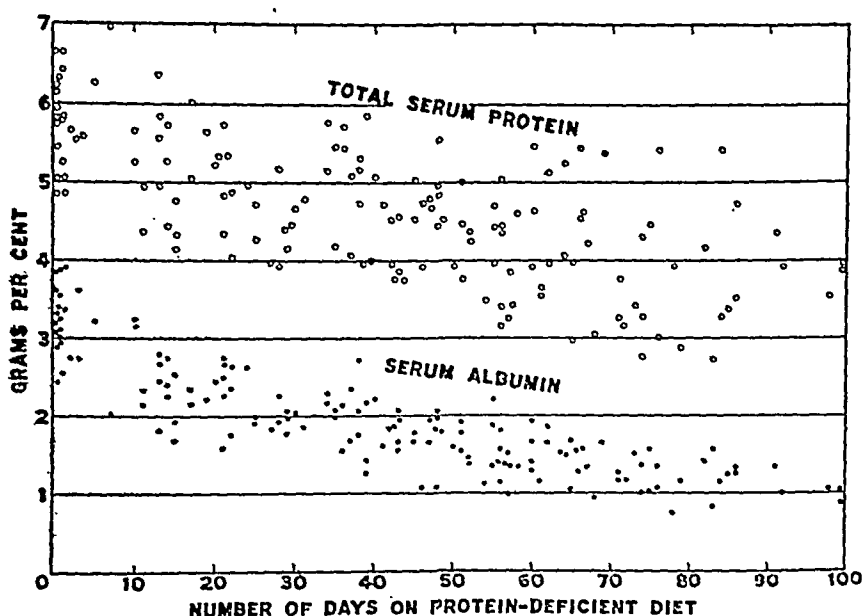


CHART 1. The results of 150 determinations of the albumin and total protein of serum in 21 dogs which were maintained on the protein-deficient diet.

significantly, higher than the initial. In a previous communication (1) we reported a tendency for globulin to rise in association with the fall in albumin. This was indeed true in a number of experiments in most of which the initial values for globulin were rather low. In other experiments, however, the initial levels were higher and the diet resulted in a decline rather than a rise. The circumstance has led us to inquire whether maintenance on the diet may not lead to greater uniformity in globulin concentrations than are encountered in normal animals. Table II presents calculations in which the possibility is

examined. Both from the standard deviations and the coefficients of variation it is seen that the globulin concentrations do tend to become less scattered and more concentrated about their averages for as long as 50 days. Thereafter the scattering again increases.

Effect of Diet on Nitrogen Balance

The protocols disclose five animals in which the nitrogen balance was followed through a total of 42 metabolism periods (approximately

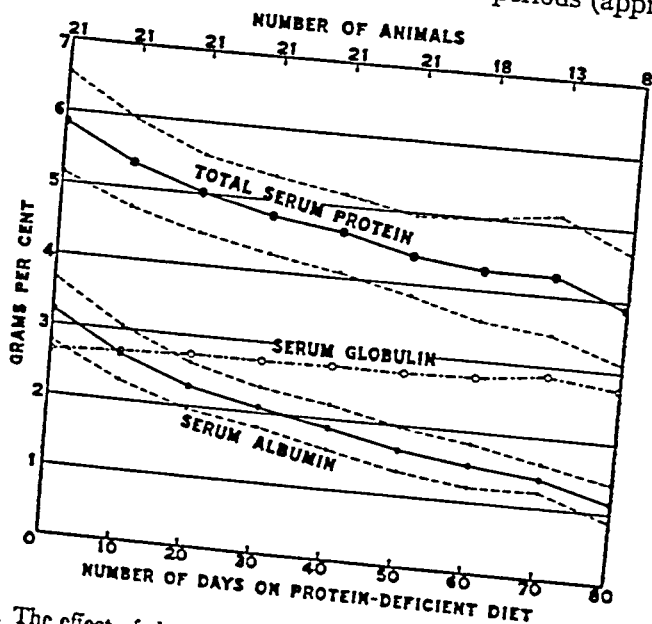


CHART 2. The effect of the protein-deficient diet on the albumin, globulin, and total protein of serum. The curves, which represent average values, were prepared from the data in Chart 1. The dotted lines above and below the unbroken lines for albumin and total protein are placed at a distance of one standard deviation from the average values. They mark out the range within which 68 per cent of the observations may be expected to fall.

294 days). In every period the excretion of nitrogen was greater than the intake. The quantities of nitrogen lost are shown in Table III. With two animals, in which the record of nitrogen balance is complete for the entire experimental period, there is seen to be a tendency toward a decrease in the amount of nitrogen excreted with continuance

of the diet. These animals lost an average of 1.77 gm. nitrogen daily during the first 2 weeks of maintenance on the diet and an average of only 0.85 gm. during the last 2 weeks. A similar progressive diminution in the nitrogen loss has been described by Liu and his collaborators (8) among patients who were subsisting on low protein diets similar to those prevalent in the famine districts of China. The adaptive ability of the body in adjusting its metabolic processes so as to spare protein is further seen from the circumstance (Table III) that the decrease in the amount of nitrogen lost has taken place in spite of a marked reduction in the quantity of food consumed. The table does reveal a rise in the loss of nitrogen with each sharp drop in the intake of food

TABLE II

Course of the Serum Globulin in 21 Dogs Maintained on Protein-Deficient Diet

Time maintained on defective diet	Average per 100 cc.	Standard deviation of distribution	Coefficient of variation
<i>days</i>	<i>gm.</i>		
Start	2.65	0.77	29.1
10	2.69	0.63	23.3
20	2.72	0.52	19.2
30	2.72	0.50	18.4
40	2.75	0.49	17.7
50	2.74	0.46	16.9
60	2.78	0.52	18.6
70	2.88	0.67	23.4
80	2.74	0.70	25.7

but the rises are not sufficient to compensate for the general progressive decline in nitrogen excretion. The average daily loss of nitrogen for all of the animals studied was 1.15 gm. It is evident that the figure would be greater if more animals had been studied during the initial weeks of maintenance on the diet.

Relation between Negative Nitrogen Balance and Decline in Serum Albumin

The data presented in the preceding sections permit an estimate of the relationship between loss of albumin from the circulation and loss of nitrogen from the body as a whole. The average initial weight of

the five dogs whose nitrogen balance was studied was 16.8 kilos. If the average volume of the plasma is taken to be 50 cc. per kilo, and the average concentration of albumin in the serum is 3.2 gm. per cent, it follows that the average circulating albumin with these animals was initially 27 gm. After 30 days of maintenance on the diet, when the

TABLE III
Nitrogen Balance and Food Consumption in Five Dogs Maintained on Protein-Deficient Diet

Time on diet	Dog 8-06 15.45 kg.		Dog 8-4a 15.55 kg.		Dog 8-38 20.60 kg.		Dog 8-4b 18.00 kg.		Dog 2-3 14.15 kg.	
	Food* consumed per day per kg.		Food consumed per day per kg.		Food consumed per day per kg.		Food consumed per day per kg.		Food consumed per day per kg.	
	calories	Daily† loss of nitrogen gm.	calories	Daily loss of nitrogen gm.	calories	Daily loss of nitrogen gm.	calories	Daily loss of nitrogen gm.	calories	Daily loss of nitrogen gm.
days										
1-8	77.7	1.76	77.2	2.02	—	—	—	—	—	—
8-15	77.7	1.52	77.2	1.77	58.3	—	66.7	—	84.8	—
15-22	77.7	1.42	77.2	1.59	58.3	—	66.7	—	84.8	—
22-29	77.7	1.39	77.2	0.85	58.3	—	66.7	—	84.8	—
29-36	77.7	1.42	64.8	1.06	58.3	—	66.7	—	84.8	—
36-43	67.2	0.92	72.4	0.97	55.0	—	66.7	—	84.8	—
43-50	34.7	1.36	54.4	1.10	36.0	1.49	66.7	—	84.8	—
50-57	38.9	1.34	57.8	0.88	39.3	1.79	66.7	—	84.8	0.40
57-64	38.9	0.97	51.7	1.07	27.5	1.76	60.5	—	84.8	0.70
64-71	38.9	1.02	37.2	1.13	29.2	1.48	32.5	—	84.8	0.80
71-78	32.2	1.08	24.8	0.77	29.2	1.38	33.4	0.77	82.8	0.54
78-85	38.9	0.90	18.6	0.79	23.2	0.42	31.4	0.96	55.8	0.81
85-92	38.9	0.94	—	—	—	1.28	19.5	1.60	33.6	1.04
Average.....	1.23	—	1.17	—	1.37	—	1.11	—	28.8	1.22
										0.79

* The food consumption per kilo was calculated on the basis of body weight at the start of each experiment.

† Loss of nitrogen represents the excess of excretion (nitrogen in urine plus nitrogen in stool plus nitrogen in washings from cage) over intake (nitrogen in diet).

concentration had been reduced to 2.1 gm. per cent, the total albumin in the circulation was 18 gm. This means a loss of 9 gm. albumin or 1.4 gm. nitrogen from the circulation. During the same 30 days the dog lost at least 1.15 gm. nitrogen daily from its entire body, or a total of 35 gm. It is, therefore, seen that the loss of nitrogen from the

circulation was only 4 per cent of the loss from the organism as a whole. A similar calculation based on the first 60 days of maintenance on the diet shows only 3 per cent of the total nitrogen loss accounted for by the decline in albumin in the serum. For purposes of simplicity we have omitted from the calculations consideration of the decline in plasma volume which has been reported to accompany plasma albumin deficits. In our experience the decline averages around 20 per cent of the initial volume and the error introduced by simplifying the calculation would seem to be compensated by the low figure used for total

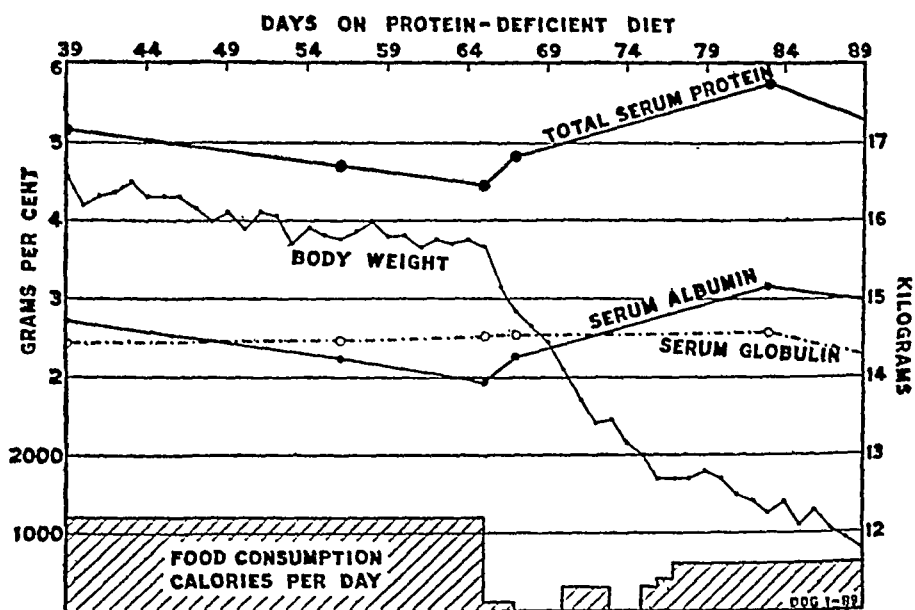


CHART 3. Spontaneous regeneration of the albumin of serum associated with partial fasting and rapid loss of weight.

daily nitrogen loss. At all events it is desired merely to stress the discrepancy which exists between serum loss of nitrogen and total loss of nitrogen, a discrepancy which must remain after refinements of computation.

Spontaneous Regeneration of Plasma Protein at Expense of Tissue Protein

In the majority of experiments, as has been described, depletion of the tissue proteins has been accompanied by gradually declining

plasma proteins. One episode, however, has demonstrated that these processes need not always go hand in hand. The details of the episode are shown in Chart 3.

During the first 64 days of maintenance on the diet the dog reacted similarly to other animals and the serum albumin was gradually depleted to a level of 1.95 gm. per cent. On the 65th day, simultaneously with the appearance of a mild edema about the Achilles tendons, the dog suddenly refused to eat. The anorexia was not associated with fever or other evidence of infection. For about 10 days fasting was complete except for small amounts of sugar and ample amounts of salt and

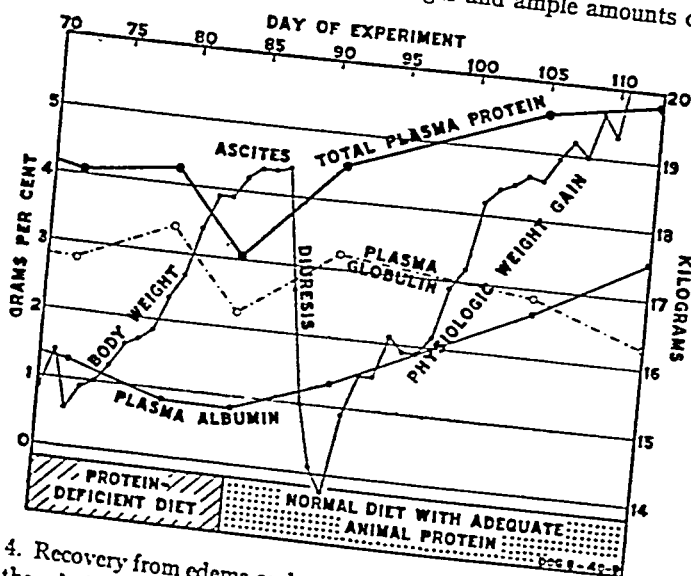


CHART 4. Recovery from edema and restoration of the serum proteins to normal following the administration of a diet containing adequate protein.

water which were given by gavage. Finally after the 76th day smaller amounts of the previous diet were administered by force. During the interval of approximate fasting the animal lost about 3 kilos. A small portion of the loss may have resulted from the elimination of interstitial fluid but the greater part must have represented consumption of tissue for energy needs. There was certainly a considerable loss of nitrogen. Under the circumstances the course of the serum proteins was astonishing. The albumin rose to a level of 3.17 gm. per cent, a value within the range of normal, and the globulin remained constant at a normal level. During this period there was no evidence of dehydration and in fact the change in the serum proteins which involved a shift in the albumin:globulin ratio, could not have resulted from blood concentration alone.

We have been forced to believe that the serum albumin in this animal was regenerated at the expense of catabolized tissue protein. The belief is strengthened by the subsequent course of events, when the dog began to eat voluntarily, and the serum albumin again declined, not acutely, but gradually, as during the early days of the experiment.

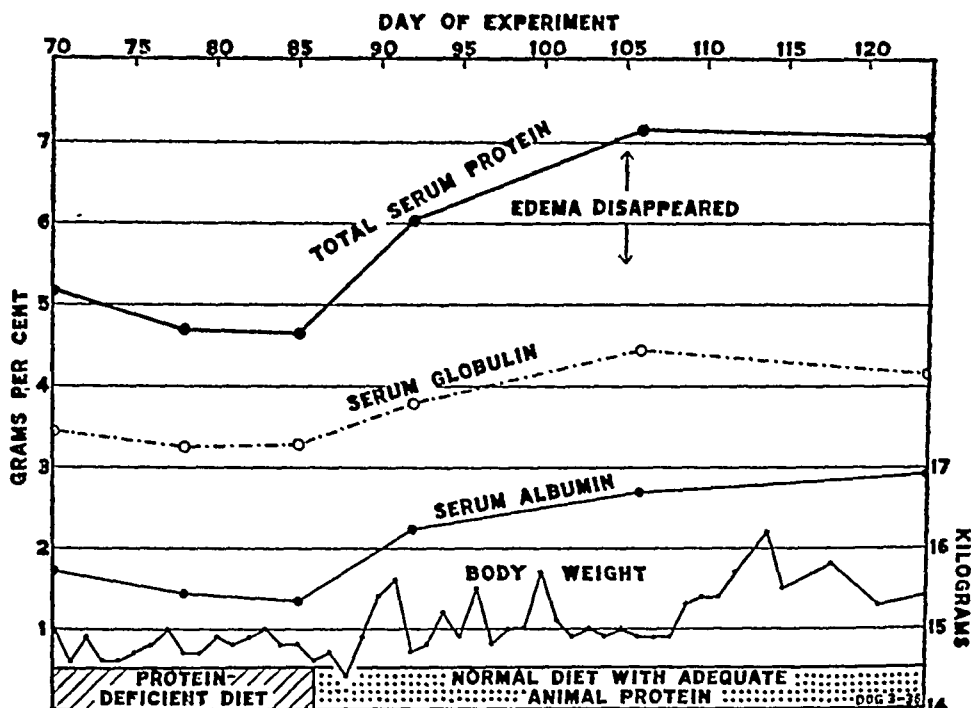


CHART 5. Replenishment of the serum proteins from feeding a diet adequate in protein in an animal in which recovery was very slow. At the start of the experiment the albumin concentration was 3.9 gm. per cent. The lowest level recorded was 1.4 gm. per cent on the 85th day. After 38 days of adequate feeding it had again risen to 2.9 gm. per cent.

Serum Proteins during Recovery

If the process of serum protein depletion is interrupted by administering a diet containing adequate protein, it is usually possible to bring about a return to normal health. If the protein is fed before marked weakness and loss of vitality have ensued, the rise in the serum albumin may be astonishingly rapid. In one experiment on a diet of

milk and meat the albumin rose from 1.1 to 2.8 gm. per cent in 10 days. In a few animals the attempt to evoke recovery was not made until the physical condition was critical and it seemed that life could not be prolonged on the low protein diet for more than a few days. Due to weakness and severe anorexia these animals may require much careful nursing before health is restored. We have usually begun by administering daily milk gavages and have added casein, lean meat, or liver to a diet composed chiefly of bread and vegetables in attempts both to provide protein and to stimulate appetite. Under this treatment an immediate rise in both the albumin and globulin fractions of serum has always been observed. The rise need not, however, be associated immediately with improvement in the physical condition and some dogs have died at this stage. In others restoration of vitality and return of the serum proteins to normal levels occurs but may be an extremely slow process. Chart 4 illustrates the phase of recovery in an average animal. In this experiment the serum proteins had returned to normal levels after 29 days on a diet containing added milk, meat, and liver consecutively. Chart 5 outlines an experiment in which restoration of the normal blood protein pattern was greatly delayed. With this animal feedings by gavage were necessary for many days and even after 38 days of adequate protein intake, the period embraced by the chart, the serum proteins were still appreciably removed from the previous level of health.

Charts 4 and 5 both show that the feeding of adequate protein is followed immediately by a rise in serum globulin as well as in albumin. The regeneration of globulin often leads to concentrations which are above normal. Subsequently as the albumin continues to rise the globulin again sinks until the normal ratio is reestablished. This experience has been a general one. The course of events is quite analogous to that described by Weech and Ling (9) in human beings during recovery from the hypoproteinemia of famine edema.

Our experiments have yielded no clue as to which of the several dietary proteins produced the most rapid regeneration of serum protein. It is generally agreed that animal protein is more suitable than vegetable protein and for this reason we have employed protein of animal origin only. Between the several proteins there has been little to choose. We have felt that progress was more satisfactory

when the individual animal's whims of appetite were catered to than when persistently trying the effect of this or that type of protein. In a subsequent paper it will be shown that dogs deprived of protein for long periods present histologic and symptomatic evidence of liver damage. It is possible that replenishment of the serum proteins is determined not only by adequate diet but also by the time required for restoration to anatomic and physiologic normality of those organs which are concerned in the synthesis of serum protein.

DISCUSSION

In a recent publication Bloomfield (10) has brought together a number of reasons for suggesting that loss of protein in the urine and lack of protein in the diet are not in themselves sufficient to account for the phenomenon of hypoproteinemia. This he refers to as the inadequacy of "the loss and lack theory." More recently Holman, Mahoney, and Whipple (11) have suggested the existence of a "dynamic equilibrium between tissue protein and plasma protein" which is governed by "the physiological needs of the moment." Inasmuch as an understanding of the nature of an equilibrium would probably go far toward explaining the inadequacies of the loss and lack theory, it appears worth while to summarize the evidence for and against the equilibrium concept.

A. In favor of the existence of an equilibrium between tissue protein and plasma protein, the following facts may be cited:

1. The studies of nitrogen balance reported in this paper show that the depletion of the plasma protein in dogs which results from maintenance on a low protein diet is accompanied by a much larger loss of protein from the tissues.

2. The common forms of hypoproteinemia in the human being are accompanied by malnutrition and asthenia which are probably indicative of tissue protein deficit. The amount of nitrogen retained during recovery from human nutritional edema far exceeds the quantity required to restore the plasma proteins to normal levels (8, 12). One of the patients studied by Schittenhelm and Schlecht retained 16 gm. out of 21 gm. of nitrogen ingested in a single day.

3. In plasmapheresis experiments sudden withdrawal of plasma protein from the animal is followed by the regeneration of a new

supply even though the animal be fasting (13). With repeated plasmapheresis over a number of days the reduction in plasma-protein is small in comparison with the volume of blood which must be exchanged. In one experiment in this laboratory conducted over a period of 12 days it was estimated that each 4 gm. of albumin withdrawn from the circulation effected a reduction of only 1 gm. in the circulating amount and that 7.5 gm. of globulin were removed for each gram of reduction in this fraction. Moreover, the attained degree of hypoproteinemia was temporary and followed by further regeneration when the bleedings were stopped.

4. The nitrogen requirement of the tissues can be met by suitable amounts of plasma protein injected intravenously. With such injections Holman, Mahoney, and Whipple (11) have shown that dogs receiving only sugar by mouth can be maintained "practically in nitrogen equilibrium." Large amounts of injected protein do not produce abnormally high concentrations in the recipient, the protein being removed apparently to supply tissue needs.

5. Analogies exist which show that under stress there may be an interchange of protein-building material between other tissues in the body. Davis and Whipple (14) demonstrated that dogs which exhibit hepatic necrosis from poisoning with chloroform can regenerate the liver back to normal on an intake of sugar alone.

B. In conflict with the concept of an equilibrium between tissue protein and plasma protein, the following facts may be cited:

1. The experiment illustrated in Chart 3 shows that tissue protein and plasma protein do not always move in a parallel manner and that occasionally the plasma protein may rise while the tissue protein falls in a way which appears to be at complete variance with the existence of an equilibrium between the two.

2. In these experiments there has been no apparent relationship between the original state of nutrition and muscular development of dogs and the rapidity with which they developed hypoproteinemia. When the experimental program was started it was supposed that edema levels might be reached more quickly if the initial nutritional status was poor. The supposition was in error. The results are more satisfactory when normally nourished vigorous animals are chosen. In the experiment referred to in the preceding paragraph the rate of

decline in the plasma protein following the episode of spontaneous regeneration was not more rapid than is observed in normal animals *de novo*.

3. During recovery from nutritional edema, both in man and in the dog (15), the plasma proteins may regenerate to normal levels in less time than is required for the restoration of normal nutrition.

4. Although experience with human beings shows that plasma protein deficits are generally associated with malnutrition, the reverse is not true and severely malnourished individuals are frequently encountered in whom the plasma proteins have a normal level.

5. Complete fasting is usually not attended by significant reduction in the concentration of protein in the plasma (13, 16). The explanation, that fasting does lead to a loss of protein from the circulation but that concentration is maintained at the expense of reduction in blood volume, is not satisfying as it is difficult to think of an equilibrium between tissue protein and plasma protein which depends upon total quantities rather than upon concentrations.

6. Bloomfield (10, 17) has shown that rats maintained on various synthetic diets of low protein content or on a diet composed of dry carrot powder do not exhibit progressive hypoproteinemia, whereas on a diet of fresh carrots the phenomenon does ensue. In both groups loss of weight was interpreted as evidence that lack of protein had rendered the diets inadequate for maintenance. Bloomfield suggested that difference in water content of the diets might be the determining factor. In any case the experiments indicate again that loss of tissue protein and loss of plasma protein do not always go hand in hand. Because of the importance of Bloomfield's observations it is to be hoped that figures showing the nitrogen balance will eventually be supplied to provide final proof of loss of protein, as opposed to fat and possibly glycogen, from the body. We may note in passing that progressive hypoproteinemia has been observed in this laboratory to develop in dogs maintained on a synthetic diet somewhat similar to that employed by Bloomfield.

In the present state of knowledge it is impossible to reconcile all of the facts presented above. It has seemed desirable to list them merely because in so doing issues are rendered apparent which may become the starting point for new experiments. It is clear that the

possibility of exchange of substance between tissue protein and plasma protein does exist and that under appropriate stress either protein may act as a restorative for the other. It is not clear, however, that the exchange results from a direct equilibrium between the two proteins. Under circumstances which are not understood, tissue protein alone may suffer when the nitrogen balance is negative and when both proteins have been depleted tissue protein may suddenly, and for totally obscure reasons, suffer additional losses which permit the replenishment of plasma protein. Because in many instances a restorative action of this nature is in abeyance, it is not justifiable to postulate injury of the blood protein-forming mechanism. The mechanism may fail simply for lack of raw material which must be supplied from other sources.

In spite of many points the nature of which remains obscure, the data presented in this paper support the contention of Bloomfield that the loss and lack theory alone is an inadequate explanation of hypoproteinemia. We have hoped that some of the data might carry therapeutic implications. So long as the theory is believed there can be little justification for attempting to restore the plasma proteins by other means than the feeding of adequate protein. The physician, and particularly the pediatrician, knows how difficult such feeding may be in the face of severe anorexia and when attempts to force the diet regularly lead to vomiting. At the present time it is not unjustifiable to hope that a way will be found for stimulating an internal readjustment to provide temporary relief, a way which will depend upon the potential ability of the tissues to provide sufficient protein for the needs of the plasma.

SUMMARY

1. The concentration of protein in the serum and plasma of normal dogs is given. Analyses of serum from 38 animals yielded the following averages and standard deviations, (a) for albumin: 3.26 ± 0.48 gm. per cent, (b) for globulin: 2.72 ± 0.76 gm. per cent, and (c) for total protein: 5.98 ± 0.67 gm. per cent. Analyses of plasma from 19 animals showed, (a) for albumin: 3.38 ± 0.38 gm. per cent, (b) for globulin: 2.98 ± 0.55 gm. per cent, and (c) for total protein: 6.36 ± 0.71 gm. per cent.

2. A diet for dogs is described, the feeding of which results in a progressive decline in the concentration of protein in the serum. A composite curve constructed from the findings with 21 animals discloses a rapid initial fall and a slower subsequent decrease in albumin and total protein and an approximately constant level for globulin. The course of the globulin curve was subject to wide variation in separate experiments, both increases and decreases being recorded.

3. With five dogs the nitrogen balance was followed through a total of 42 metabolism periods of approximately 7 days each. The average daily loss of nitrogen was 1.15 gm. Approximate calculations disclose that only 3 or 4 per cent of the nitrogen eliminated is accounted for by the decline in circulating protein, the remainder being represented by loss from the tissues.

4. An episode is described with one dog when, during a period of self-imposed fasting, the serum albumin regenerated to a normal level, apparently at the expense of catabolized tissue protein.

5. The course of serum proteins is described during the recovery which follows interruption of the low protein diet and return to a régime of adequate feeding.

6. A discussion is given of the relationship between tissue proteins and plasma proteins. The data permit one to entertain the hope that a way will be found for stimulating an internal readjustment to provide temporary relief from hypoproteinemia, a way which will depend upon the potential ability of the tissues to provide sufficient protein for the needs of the plasma.

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RENAL DAMAGE FOLLOWING THE INGESTION OF A DIET CONTAINING AN EXCESS OF INORGANIC PHOSPHATE

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PLATES 12 TO 14

(Received for publication, November 15, 1934)

The addition of an excess of acid or basic sodium phosphate to the food of young albino rats is followed (1) by a remarkable increase in the size and weight of the kidneys. The gross appearance of these organs is not entirely normal and histological examination discloses extensive pathological changes which have resulted from the administration of inorganic phosphate (2). A description of this renal lesion and a series of experiments which demonstrate that it is due to a specific effect of the phosphate ion form the subject of the present communication.

Methods

The general experimental procedure has been described in detail elsewhere (3). Female albino rats were used as the experimental animal. In each case they were removed from the mother and placed upon the experimental diet when exactly 26 days of age. 44 days later, that is, when the rats were 70 days old, they were etherized and killed. Anatomical measurements were then made in the manner which has been described. Although changes in renal weight were the main interest, the weights of the heart and liver were also recorded. The kidneys and pieces of the heart and liver were preserved in Orth's fluid for histological examination.

Throughout each experiment every rat was weighed daily. If there was a loss of weight for several days the rat was removed from the experiment for it then became questionable whether or not its food intake, and hence the quantity of ingested phosphate, was comparable with that of others of the group. The daily food intake per rat was determined by measuring the amount of food consumed by groups of 4 to 6 rats over 2 day periods.

Experimental Diets

Experiments were carried out with nine different diets containing inorganic phosphate in five different forms. All of the diets were identical in so far as their protein, fat, carbohydrate, basic mineral and vitamin-containing food content were concerned. Their caloric value was also identical. They differed only in the amount and type of inorganic phosphate which they contained. 90 per cent of these diets was composed of the mixture described in Table I. The remaining

TABLE I
Control and Basal Diet

	<i>per cent</i>	
Corn-starch.....	41.0	
Casein.....	15.0	
Lard.....	15.0	
Cod liver oil.....	9.0	
Salt mixture (Osborne and Mendel).....	4.0	
Dried yeast.....	4.0	
Wheat germ.....	10.0	
Agar-agar.....	2.0	
1 gm.—4.8 calories		
Element	Per cent of diet	Per cent of calories
Protein.....	17.2	14.8
Fat.....	25.1	48.6
Carbohydrate.....	42.7	36.6
Water.....	6.3	0.0
Roughage.....	3.4	0.0
Salts.....	5.2	0.0

constituents of each of the diets are summarized in Table II. The phosphate content and changes in the sodium and potassium contained in each diet are given in Table III. Diet 2 is strongly acid and acid-producing and Diet 5 strongly alkaline before and after being metabolized. Diet 3 is definitely acid and No. 4 definitely alkaline while the remainder are more nearly neutral. From 18 to 25 rats comprised the group which was fed upon each diet. Since more than 3 years intervened between the first (Nos. 1, 3 and 4) and the remaining phosphate experiments, two control experiments were carried out

on Diet 1, one (No. 1) with the original group and a second (No. 1a) 3 years later. They gave essentially the same results and are evidence of the constancy of the experimental conditions.

TABLE II

Experiment No.	Agar-agar	H ₃ PO ₄	NaH ₂ PO ₄	Na ₂ HPO ₄	Na ₃ PO ₄	KH ₂ PO ₄	K ₂ HPO ₄
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	10.00						
2	6.54*	2.94					
3	4.50		5.50				
4	3.50			6.53			
5	1.70*				4.92		
6	6.07		1.80	2.13			
7	4.10		2.71	3.19			
8	2.14		3.60	4.26			
9	5.35					2.04	2.61

* These ingredients do not total 10 per cent because of water in the phosphate compound which made up the difference.

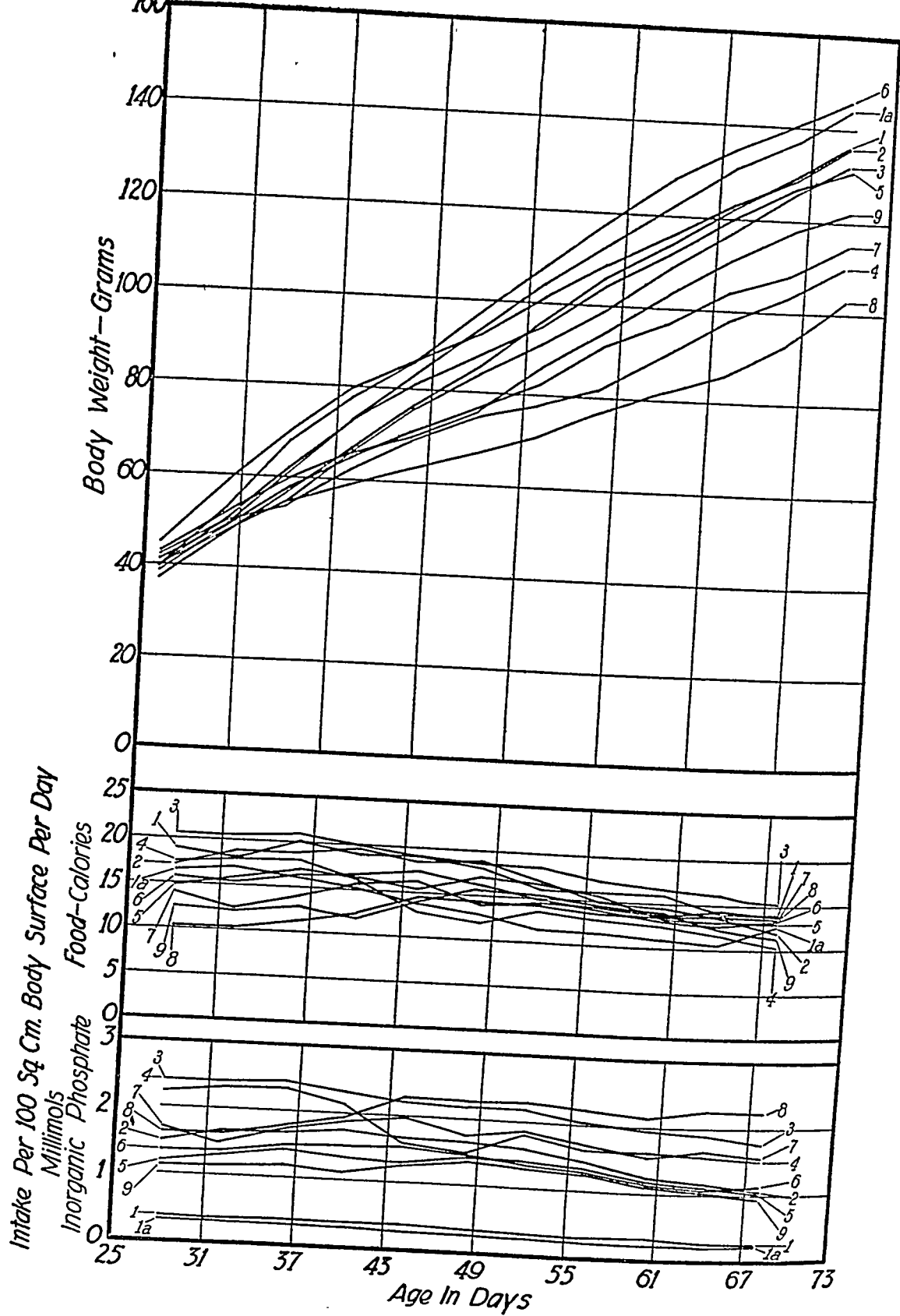
TABLE III

Experiment No.	Form of added phosphate	Added phosphate	Total phosphate	Added sodium	Total sodium	Added potassium	Total potassium
		Per 100 gm. food					
		<i>m.-eq.*</i>	<i>m.-eq.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>
1			20.0		5.3		12.9
2	H ₃ PO ₄	90.0	110.0		5.3		12.9
3	NaH ₂ PO ₄	138.0	158.0	46.0	51.3		12.9
4	Na ₂ HPO ₄	138.0	158.0	92.0	97.3		12.9
5	Na ₃ PO ₄	90.0	110.0	90.0	95.3		12.9
6	NaH ₂ PO ₄ (45) +Na ₂ HPO ₄ (45)	90.0	110.0	45.0	50.0		12.9
7	NaH ₂ PO ₄ (67.5) +Na ₂ HPO ₄ (67.5)	135.0	155.0	67.5	72.8		12.9
8	NaH ₂ PO ₄ (90) +Na ₂ HPO ₄ (90)	180.0	200.0	90.0	95.3		12.9
9	KH ₂ PO ₄ (45) +K ₂ HPO ₄ (45)	90.0	110.0		5.3	45.0	57.9

* M.-eq. = milli-equivalents; phosphorus assumed to be trivalent.

RESULTS

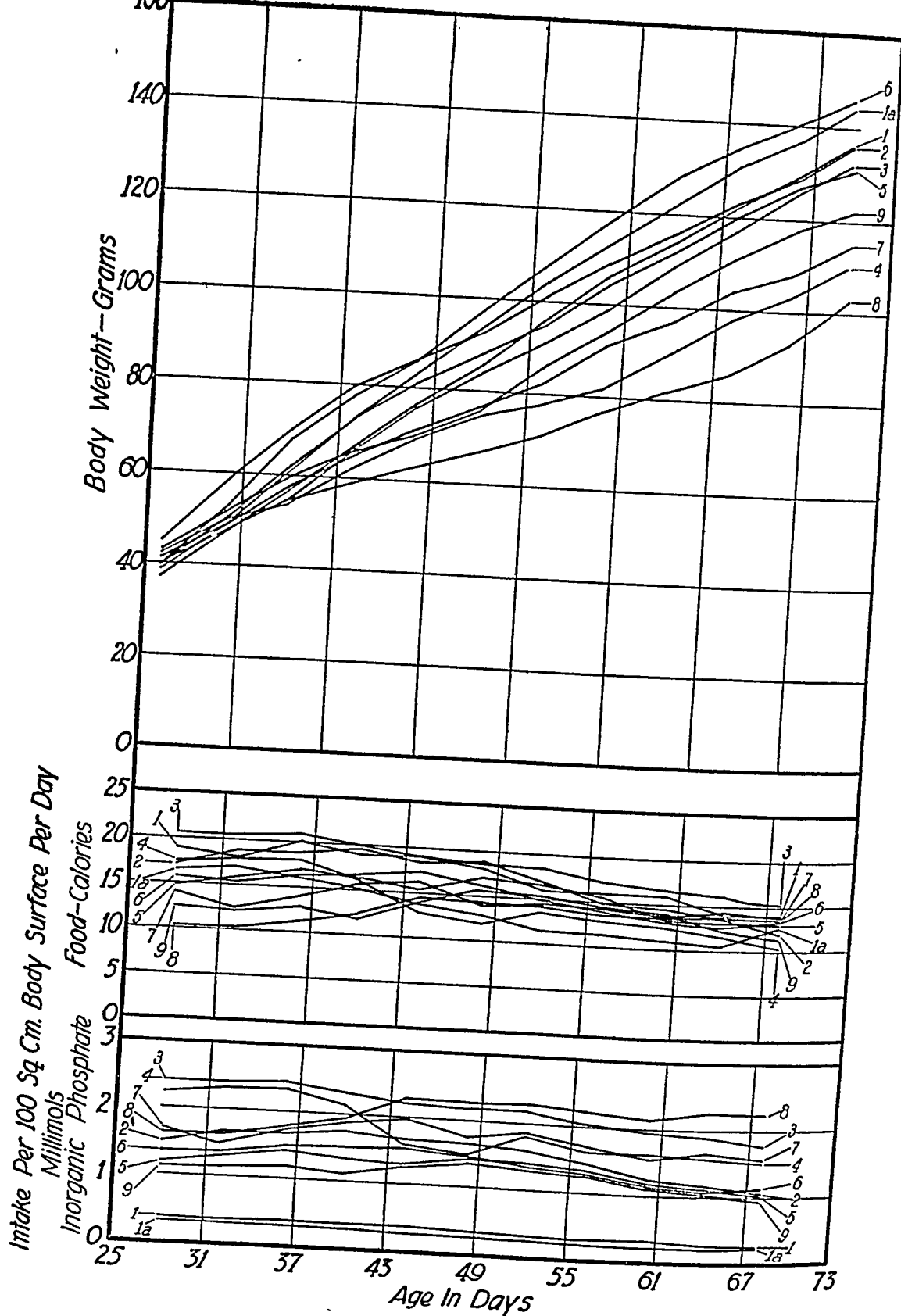
In Text-fig. 1 have been charted for each of the ten groups, growth in terms of the mean daily body weight, and food intake on the basis



TEXT-FIG. 1

TABLE IV
Group Means of Anatomical Measurements Made at Death

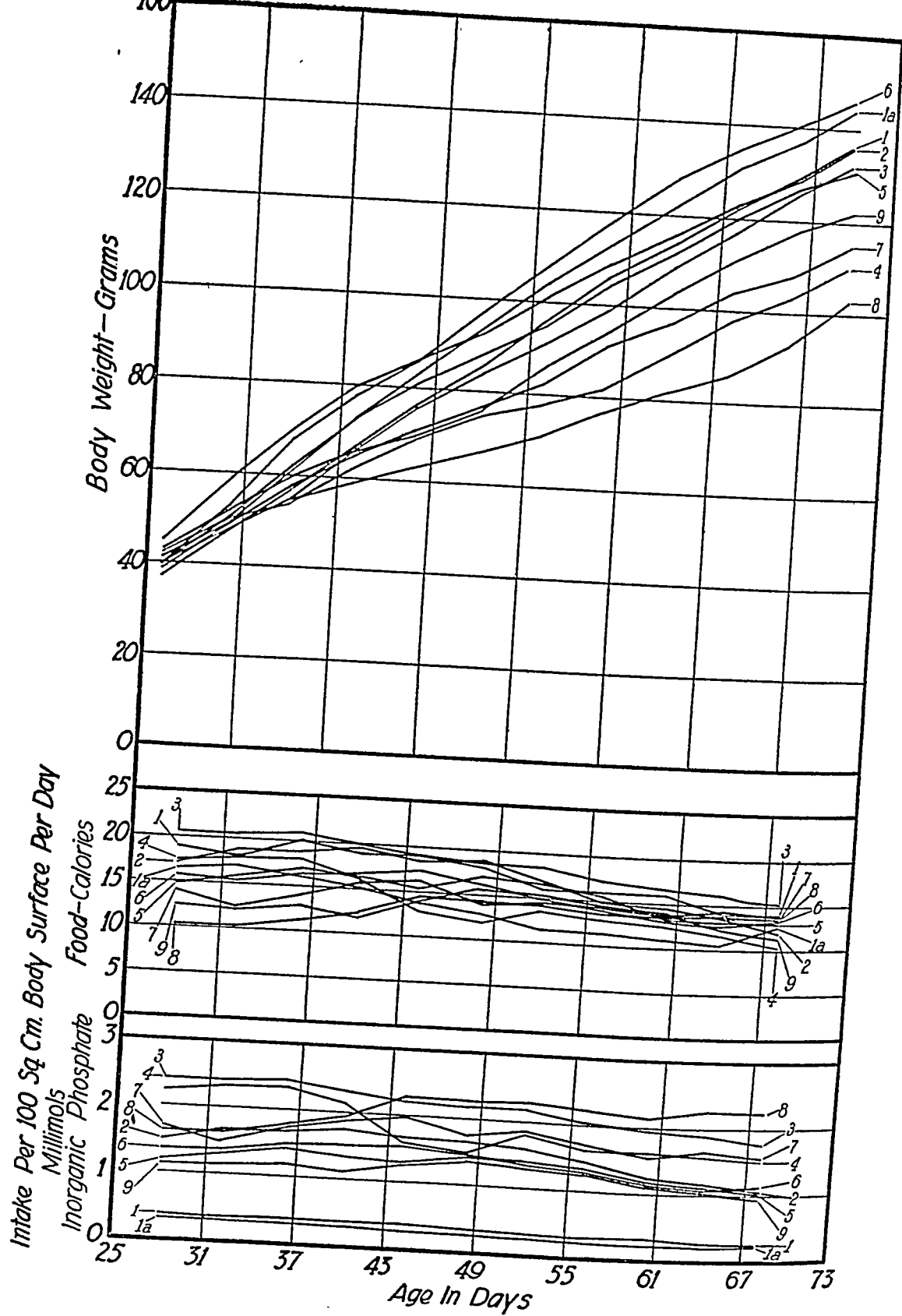
Experiment No.	1	2	3	4	5	6	7	8	9
Nature of experiment	Control	H ₂ PO ₄	NaH ₂ PO ₄	Na ₂ HPO ₄	Na ₂ PO ₄	NaH ₂ PO ₄ +Na ₂ HPO ₄	Same × 1.5	Same × 2.0	KH ₂ PO ₄ +K ₂ HPO ₄
No. of rats in experiment	25	22	24	18	22	19	22	22	22
Body weight (gross), gm.	135	135	132	111	130	137	115	97	122
Body weight (corrected), gm.	133	127	125	103	126	133	110	93	115
Body surface, sq. cm.	290	287	284	261	285	295	259	231	267
Body length, mm.	177	175	176	167	175	180	168	157	172
Heart weight (actual), mg.	501	476	516	446	493	512	452	380	464
Heart weight per 100 sq. cm. body surface, mg.	173	166	182	178	173	174	175	164	173
Liver weight (actual), gm.	5.02	5.71	6.10	5.64	5.06	5.86	4.81	4.62	
Liver weight per 100 sq. cm. body surface, gm.	2.11	1.99	2.23	2.26	1.77	1.98	1.85	2.00	
Kidney weight—left, mg.	441	653	835	848	774	821	723	689	732
Kidney weight—right, mg.	457	676	860	868	814	859	749	719	747
Kidney weight—total, mg.	898	1328	1695	1716	1588	1680	1472	1408	1479
Kidney weight—average, mg.	449	664	848	858	794	840	738	704	739
Kidney weight per 100 gm. body weight, mg.	337	523	678	834	631	631	670	757	642
Kidney weight per 100 mm. body length, mg.	254	379	482	514	452	467	439	448	430
Kidney weight per 100 sq. cm. body surface, mg.	151	233	291	342	278	288	282	303	274
Kidney weight per 100 mg. heart, mg.	90	142	165	193	169	164	162	186	159



TEXT-FIG. 1

TABLE IV
Group Means of Anatomical Measurements Made at Death

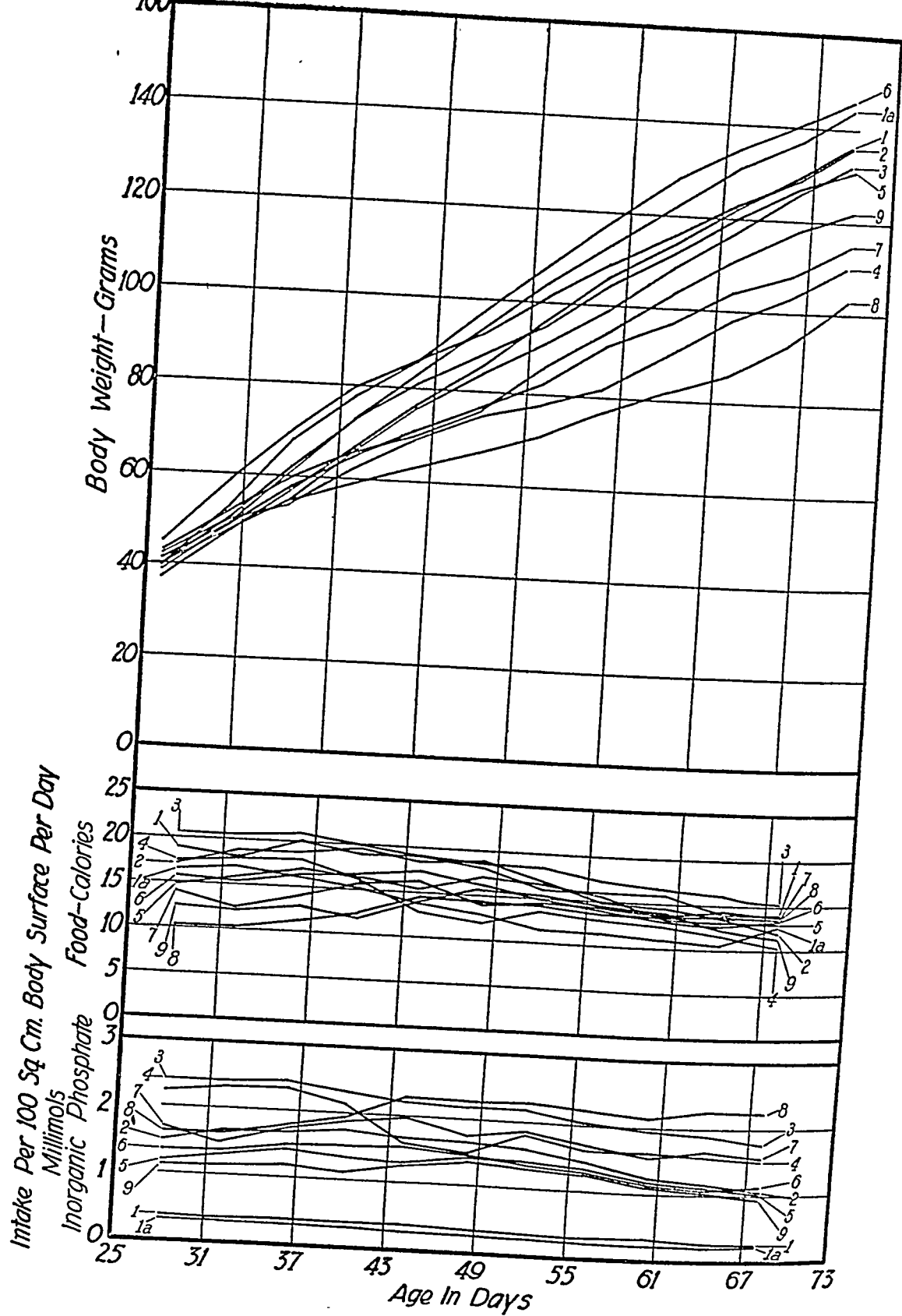
Experiment No.	1	2	3	4	5	6	7	8	9
Nature of experiment	Con- trol	Con- trol	NaH ₂ PO ₄	Na ₂ HPO ₄	Na ₃ PO ₄	NaH ₂ PO ₄ +Na ₂ HPO ₄	Same × 1.5	Same × 2.0	KH ₂ PO ₄ +K ₂ HPO ₄
No. of rats in experiment	25	22	24	18	22	19	22	22	22
Body weight (gross), gm.	135	143	132	111	130	137	115	97	122
Body weight (corrected), gm.	133	140	125	103	126	133	110	93	115
Body surface, sq. cm.	290	313	284	261	285	295	259	231	267
Body length, mm.	177	180	176	167	175	180	168	157	172
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Liver weight per 100 sq. cm. body surface, gm.	2.11	1.86	2.23	2.26	1.77	1.98	1.85	2.00	
Kidney weight—left, mg.	441	474	835	848	774	821	723	689	732
Kidney weight—right, mg.	457	502	860	868	814	859	749	719	747
Kidney weight—total, mg.	898	975	1695	1716	1588	1680	1472	1408	1479
Kidney weight—average, mg.	449	488	848	858	794	840	738	704	739
Kidney weight per 100 gm. body weight, mg.	337	348	678	834	631	631	670	757	642
Kidney weight per 100 mm. body length, mg.	254	271	482	514	452	467	439	418	430
Kidney weight per 100 sq. cm. body surface, mg.	154	158	291	342	278	288	282	303	274
Kidney weight per 100 mg. heart, mg.	90	97	165	193	169	164	162	186	159



TEXT-FIG. 1

TABLE IV
Group Means of Anatomical Measurements Made at Death

Experiment No.	1	2	3	4	5	6	7	8	9
	Con- trol	Con- trol	NaH ₂ PO ₄	Na ₂ HPO ₄	Na ₂ PO ₄	NaH ₂ PO ₄ +Na ₂ HPO ₄	Same × 1.5	Same × 2.0	KH ₂ PO ₄ +KH ₂ PO ₄
Nu. of rats in experiment	25	22	24	18	22	19	22	22	22
Body weight (gross), gm.	135	143	132	111	130	137	115	97	122
Body weight (corrected), gm.	133	140	125	103	126	133	110	93	115
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Kidney weight—right, mg.	457	502	860	868	814	859	749	719	747
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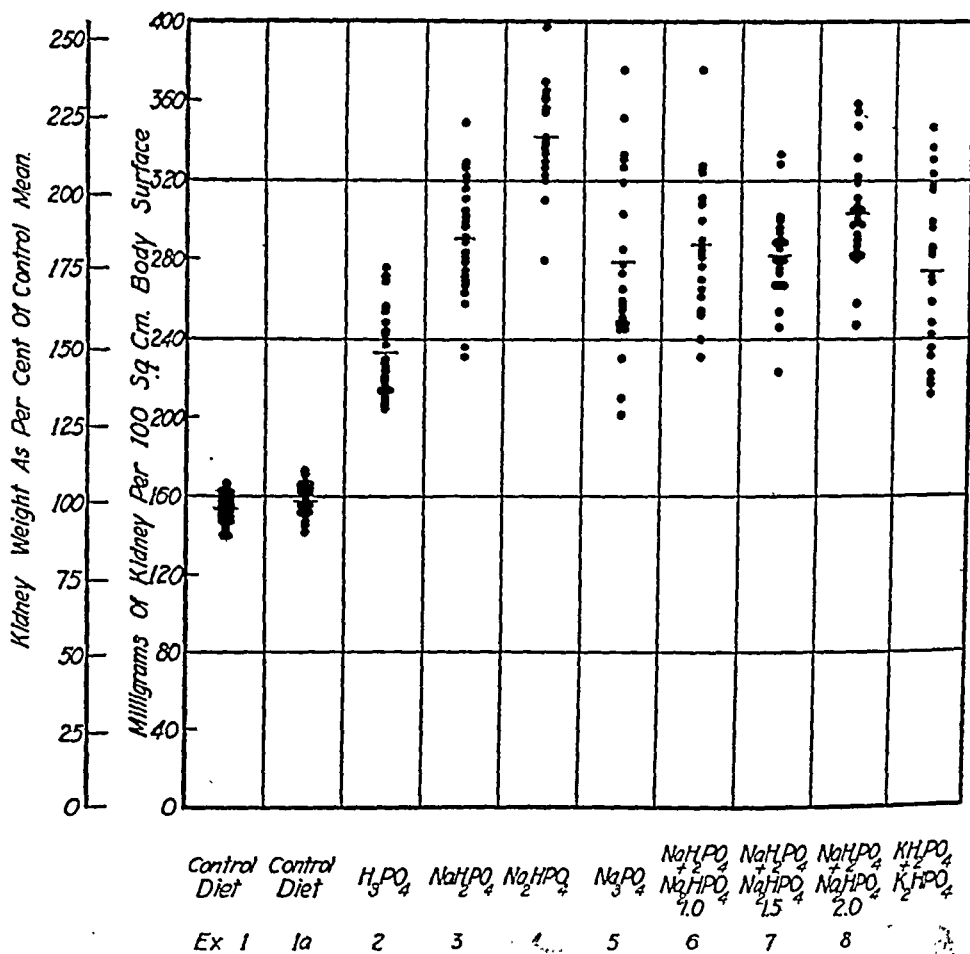
TEXT-FIG. 1

TABLE IV

Group Means of Anatomical Measurements Made at Death

Experiment No.	1	1a	2	3	4	5	6	7	8	9
Nature of experiment	Control	Control	H ₂ PO ₄	NaH ₂ PO ₄	Na ₂ HPO ₄	Na ₂ PO ₄	NaH ₂ PO ₄ +Na ₂ HPO ₄	Same × 1.5	Same × 2.0	KH ₂ PO ₄ +K ₂ HPO ₄
No. of rats in experiment	25	22	22	24	18	22	19	22	22	22
Body weight (gross), gm.	135	143	135	132	111	130	137	115	97	122
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Kidney weight per 100 gm. body weight, mg.	337	348	523	678	834	631	631	670	757	642
Kidney weight per 100 mm. body length, mg.	254	271	379	482	514	452	467	439	448	430
Kidney weight per 100 sq. cm. body surface, mg.	154	158	233	291	342	278	288	282	303	274
Kidney weight per 100 mg. heart, mg.	90	97	142	165	193	169	164	162	186	159

of the mean daily caloric intake, and phosphate consumption in relation to the calculated body surface, during the period of the experiment. Although the effect varies all of the phosphate diets had a deleterious effect upon the growth of the animals.



The mean results of the anatomical measurements made on the animals comprising Table IV. From these results it is evident that the excessive intake of phosphate in the diet leads to a considerable increase in the weight of the kidneys of the individual rats. This is particularly evident in the control group.

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From the results summarized in Table V it is seen that there is no constant relationship between the phosphate intake and the increase in renal weight. Neither is there a consistent difference in the effect of various types of phosphate. It is evident, however, from Text-fig. 3 that there is a general tendency for the increase in the weight of the kidneys to keep pace with the increasing phosphate intake. That this is not constant in the same manner as that with which the renal weight

TABLE V
Food Intake and Kidney Weight, Group Averages

Experiment No.	Nature of experiment	Intake per 100 sq. cm. body surface per day*		Kidney per 100 sq. cm. body surface		Phosphate above control	Ratio† Phosphate: Kidney weight
		Food	Phosphate	Total	Above control		
		gms.	mg.	mg.	mg.		
1	Control	3.06	0.61	154			
1a	Control	2.88	0.58	158			
2	H ₂ PO ₄	3.13	3.44	233	77	2.85	27
3	NaH ₂ PO ₄	3.54	5.60	291	135	5.01	27
4	Na ₂ HPO ₄	2.83	4.47	342	186	3.88	48
5	Na ₃ PO ₄	3.17	3.49	278	122	2.90	42
6	NaH ₂ PO ₄ + Na ₂ HPO ₄	2.91	3.20	288	132	2.61	51
7	Same as No. 6 × 1.5	3.36	5.21	282	126	4.62	27
8	Same as No. 6 × 2.0	3.30	6.60	303	147	6.01	24
9	KH ₂ PO ₄ + K ₂ HPO ₄	2.91	3.20	274	118	2.61	45

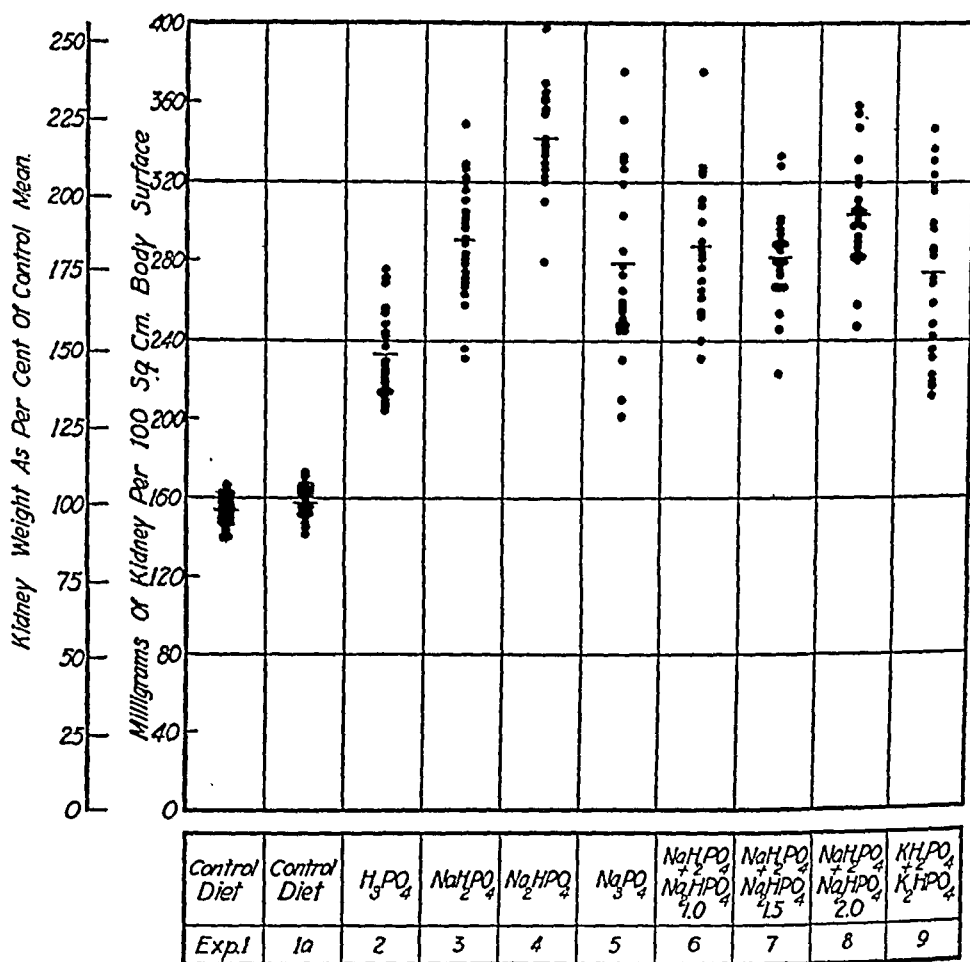
* Average of last 10 days of each experiment.

† Ratio = increased phosphate intake ÷ increase in kidney weight.

follows the protein intake (4) is hardly surprising, since a lesion is produced in this instance while the renal hypertrophy produced in increasing the protein intake is, as far as we know, physiological in nature.

The gross appearance of all of the phosphate kidneys was unusual. Their capsules always stripped readily and the color was then ordinarily more of a greyish white than the normal red-brown, particularly after the blood had been drained from the organ. In some of the experiments most of the kidneys had a smooth but mottled surface while in

of the mean daily caloric intake, and phosphate consumption in relation to the calculated body surface, during the period of the experiment. Although the effect varies all of the phosphate diets had a deleterious effect upon the growth of the animals.



TEXT-FIG. 2

The mean results of the anatomical measurements made at death comprise Table IV. From these it is evident that the addition of an excess of inorganic phosphate in any form to the diet leads to a tremendous increase in the weight of the kidneys. In Text-fig. 2 this is shown graphically for the individual rats of each group. They are likewise much larger in size than the controls (Fig. 1). The variation in the

phosphate intake had no constant effect (Table IV) upon the weight of the heart or the liver.

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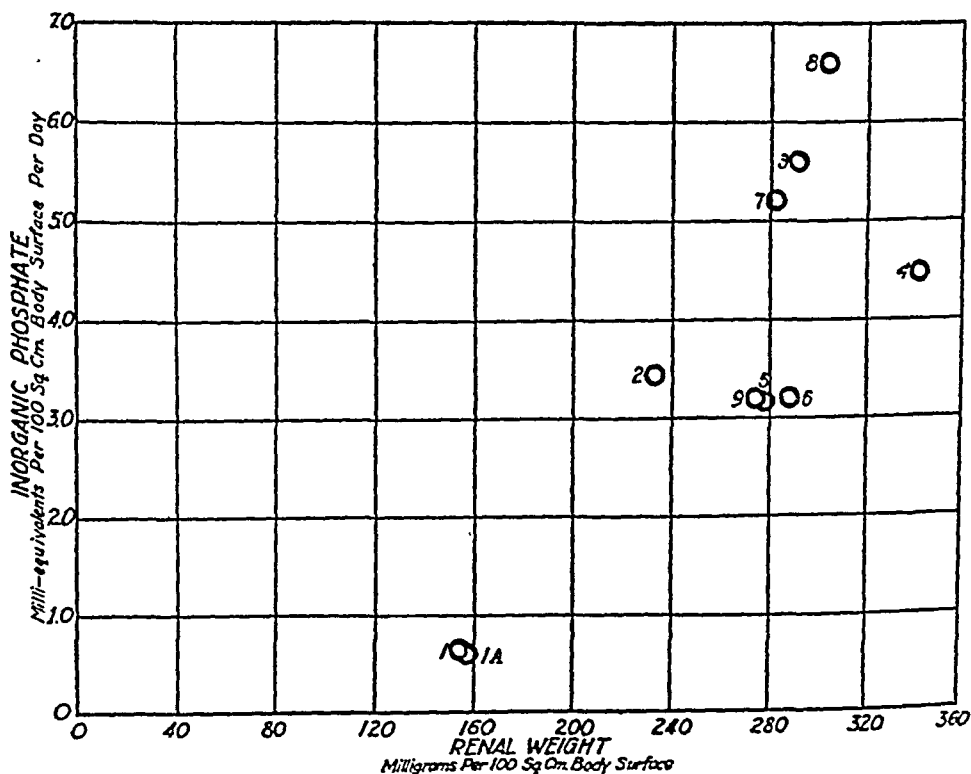
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The gross appearance of all of the phosphate kidneys was unusual. Their capsules always stripped readily and the color was then ordinarily more of a greyish white than the normal red-brown, particularly after the blood had been drained from the organ. In some of the experiments most of the kidneys had a smooth but mottled surface while in

others the majority were coarsely granular or even pebbled in appearance (Fig. 1). The cortical surfaces were most granular in Experiment 4 but also very noticeably so in Experiments 3, 7, and 8, less so in Nos. 5 and 9 and only occasionally in Nos. 2 and 6. The kidneys from all of the phosphate-fed animals felt very much firmer than normal and on section the cortex and medulla stood out clearly, particularly the outer stripe of the outer zone of the medulla.



TEXT-FIG. 3

The Histological Lesion

Microscopical examination showed essentially the same lesion in all the kidneys, irrespective of the nature of the phosphate used in the experiment. No abnormalities were found in sections of the heart or liver. The kidneys of the control animals of all the experiments were entirely normal.

The lesion consists of a complete disorganization of the outer stripe of the outer zone of the medulla. In a section of the normal rat kidney this is that clearly delimited band of tissue, lying immediately beneath the cortex which contains as its characteristic component, the terminal divisions of the proximal convoluted tubule. In the kidneys of the phosphate-fed animals it is transformed into a poorly outlined zone of distorted tubules that have lost all their normal characteristics. They are lined by a regenerated epithelium of markedly atypical appearance in which hyperplasia of the component cells is so extreme that giant cell-like masses may occlude the lumen. If an irregular lumen persists it is commonly filled with debris and masses of calcareous material. Other tubules are greatly dilated and they too may be packed with calcium deposits. About the distorted cross-sections of the tubules may be seen round cell infiltration and some fibrosis (Fig. 2).

The cortex is also involved to a greater or less degree in all the kidneys. The lesion in this part of the kidney appears to be an extension upward from the more severely involved outer stripe of the medulla and consists of either cystic dilatation of tubules or collapse of them associated with more or less round cell infiltration in the interstitial tissue. If such areas reach the free surface of the organ a retracted scar results.

The medulla, except for its above described outer stripe, is essentially normal. Occasional collecting tubules contain casts, and many of these are impregnated with calcareous substance.

The Histogenesis of the Lesion

For a study of the origin and early stages of the lesion rats were fed various mixtures of phosphate and killed on succeeding days. The earliest lesion found was observed in the kidneys of those that had been on a diet of 10 per cent Na_2HPO_4 for 1 day. The majority of the kidneys of animals of this early group show little evidence of damage, but in some the initial lesion is clearly evident and consists of a definite necrosis of the terminal portion of the proximal convolution (Fig. 3). The tubule presents a very definite localization of this damage, since it is only its termination that is involved at this early period, just at the point where the broad proximal tubule abruptly decreases in diameter to form the narrow limb of Henle's loop. The more proximal portions that extend down from the cortex are still entirely normal.

At the end of the 2nd day of feeding, similar lesions are found in the same portion of the tubule with increasing frequency. The limitation of the lesion to the termination of the tubule is, however, still observed.

During the 3rd day a rapid extension of the necrosis throughout all that part of the proximal convolution that is contained in the outer stripe of the outer zone of the medulla is regularly found in all animals. Two further complications are now present; namely, regeneration of an abnormal epithelium and the deposition of calcium salts in the necrotic debris that fills the involved tubules.

The regeneration is the most atypical that we have ever seen in the kidney. The exuberance of the process is extreme; masses of epithelial cells of varying shape and size, with large oval and vesicular nuclei heaping up on each other or fusing to form giant cells of relatively enormous dimensions replace the normal regular pattern of the outer stripe. The giant cells in many instances evidently represent the cross-section through a tubule occluded by epithelial proliferation and its center, a remnant of the lumen, may contain masses of calcareous material so that the similarity of the appearance to a typical foreign body giant cell is striking (Fig. 4).

Throughout all the damaged tubules similar calcareous deposits are found in great number (Fig. 2). They consist of scattered isolated granules and conglomerates or large spherical masses that lie either free or in necrotic debris surrounded and even infiltrated by the exuberantly regenerated atypical epithelial cells.

The further progress of the lesion can be followed through the succeeding days by the extension of the processes of necrosis, calcification and atypical regeneration throughout the entire breadth of the outer stripe of the outer zone of the medulla. Although variation is noted in the rate of development of the lesion in different experiments its general course can be described as follows:

By the 6th day the outer stripe of the outer zone is involved throughout its entire extent. Its spread towards the medulla is limited, however, and only rarely is there any extension past the sharp line which separates the inner from the outer stripe. The reason for this striking arrest of the process is evident when one remembers that the terminal portions of the proximal convolution, which are the segments of the tubule involved by the necrotizing process, stop sharply at this line of demarcation and the limitation of the lesion at this line is therefore conclusive proof that the broad ascending limbs of Henle's loop are not affected, for these extend downward through the entire

depth of the inner stripe of the outer zone of the medulla. In fact isolated ascending broad limbs lying between the degenerated and distorted proximal convolutions can still be seen in the outer zone as they ascend to enter the cortex.

By the 15th day the entire outer stripe is transformed into a zone of calcified distorted structures that bear little resemblance to tubules. Furthermore there has now developed about the distorted epithelial elements a definite increase in the interstitial connective tissue and scattered focal areas of round cell infiltration are present. Although the limitation of the spread downward through the medulla is still maintained, an involvement in the cortex becomes apparent. Necrosis and calcification along with atypical regeneration of epithelial cells has extended up the proximal convolution so that the distinctive pattern of the contiguous cortex is destroyed and blends with the abnormal tissues of the outer zone of the medulla. There results an apparent thinning of the cortex, though the cortical nature of the tissue may still be recognized by the persistence of glomeruli, still fairly well preserved, each surrounded by an island of easily recognizable proximal convolution.

Though the ascending limbs of Henle's loop never show any direct damage in the earlier stages of the lesion, it is plain that as the lesion develops they could hardly preserve their normal condition while passing through a wide band of disorganized tissue. This becomes especially true when connective tissue and round cell infiltration begins to develop in this region about the distorted tubules. The effect on the hitherto normal ascending limbs in the area of damage, *i.e.* in the outer stripe of the medulla, is collapse and their resulting disappearance as tubular structures. An even more striking effect is noted, however, at a distance from the immediate zone of damage, namely in the outer levels of the cortex, for here the distal convolution collapses as a result of the obliteration of its more proximal ascending limb of the loop. About these collapsed distal convolutions, that lie in the region of glomeruli, there occurs a proliferation of interstitial tissue and accumulation of round cells and fibroblasts and if near the surface a retracted pitting scar is thus produced (Fig. 5). Even in later stages the glomeruli persist, relatively little changed, except perhaps for occasional cystic dilatation of Bowman's space, and about

each one lies the periglomerular cluster of the attached proximal convolution also still fairly well preserved.

The final picture is therefore that of a kidney with a narrow cortex throughout which are scattered focal areas of tubular collapse and cellular infiltration and scarring. This cortex is separated by a zone of disorganized tissue, in which atypical regeneration, calcification and fibrosis are extreme, from a medulla that, except for the presence of occasional casts in the collecting tubules, is essentially normal. This destruction of the architecture of the organ is apparently a permanent one, as animals after 20 days of phosphate feeding were placed on a normal control diet with no excess of phosphate and killed after 30 days. Their kidneys still showed the lesion in a marked form.

DISCUSSION

There can be no doubt but that the renal lesion with which we are concerned is due solely to the excess of phosphate ion in the food. The changes occur in the kidney whether the phosphate is administered as the free acid, in combination with an excess of alkali (trisodium phosphate), as the acid salt (monosodium phosphate), as the alkaline salt (disodium phosphate), as a neutral mixture of these salts or whether the cation in combination with the phosphate is sodium or potassium.

Numerous cations, particularly the heavy metals, have long been known to cause varying degrees of renal damage. There is some evidence (5) that a general excess of acid radicles may result in a mild degree of renal irritation. In 1878 Kober (6) found that strong phosphoric acid given either intravenously or *per os* resulted in an acute renal irritation, but gives no evidence nor suggests that the effect was due to the fact that not only acid but phosphate had been given. Hirsch (7) describes acute changes in the convoluted tubules following the intravenous injection of sodium acid phosphate solutions which resemble very closely the renal damage which Seegal (5) found to follow a severe experimental acidosis. These changes apparently have nothing in common with the lesion with which we are concerned here.

Cramer has recently reported (8) renal lesions not unlike those with which we are dealing produced in rats fed on a diet deficient in magnesium. However the salt mixture which he used in his diet contained sufficient phosphate to produce damage and he notes that there were also many changes in the kidneys of his controls. We have been unable to produce the lesion solely through a low magnesium diet and Gough, Duguid and Davies (9) have reported a similar experience. These

investigators in a way have confirmed our report (2) of phosphate damage to the kidneys, for in studying the renal lesions of hypervitaminosis D they found that the damage was greatly intensified when large amounts of sodium phosphate were added to the diet. The kidneys from these rats showed lesions very similar to our findings.

Our experiments give no evidence concerning the mechanism by which the renal lesion is produced in the administration of phosphate. A direct effect of an excess of phosphate ions on the renal cells seems the most likely mechanism, although we have been able to produce only a transient swelling of the tubular epithelium through daily intra-peritoneal injections of a neutral sodium phosphate solution in a quantity equal to that which produces marked renal damage when given *per os*. The deposition of calcium salts seems definitely to follow cell destruction, a point that has given rise to speculation in the rather similar lesions produced by parathyroid extract (10, 11) and toxic doses of viosterol (12, 13). The calcification would seem therefore to be analogous, though much more excessive, to the commonly observed deposition of calcium in the necrotic tubular debris that results from damage by many renal poisons. Even the degree observed in our experiments, however, may be equalled in the lesion caused by toxic effects of such a different substance as arsphenamine (14).

The anatomical aspects of the lesion are interesting, chiefly from two standpoints. First, in the very definite localization of the damage to a certain region of the kidney. The lesion begins in and, except for certain secondary effects, remains localized to the outer stripe of the outer zone of the medulla. This localization depends in turn on the fact that it is only the terminal segment of the proximal convoluted tubule that is directly affected by the toxic agent. Selective action of renal poisons on various parts of the proximal convolutions are well known but in no case, not even after uranium poisoning (15) do the late changes in the architecture of the kidney remain so localized. The second point of interest is the speed with which the lesion develops. By the end of the 3rd day, the outer stripe of the outer zone of the medulla is completely transformed, not only by the regressive processes of degeneration and necrosis but also by the proliferative changes of regeneration and hyperplasia.

SUMMARY

The addition of an excess of inorganic phosphate in the form of orthophosphoric acid, acid, basic or neutral sodium or potassium phosphate to the diet of albino rats results in the development of an interesting and permanent renal lesion.

The phosphate renal lesion is characterized by a necrosis of the cells of the convoluted tubules commencing at the terminal end, followed by a regeneration of atypical epithelium and calcification of the necrotic debris that fills the tubules.

The entire outer stripe of the outer zone of the medulla is transformed into a zone of distorted structures and there is an increase in the interstitial connective tissue. The adjoining cortex is also involved with cystic dilatation of tubules and collapse. Such areas may reach the free surface of the organ and produce a retracted scar.

In the gross the kidneys are enlarged and firm on section with a pebbled surface produced by numerous scars.

The maximum changes in the kidney structure are reached after some 15 days although necrosis of the convoluted tubule cells is evident after a single day of phosphate feeding.

The renal structure is not restored to its normal form when the excess of phosphate is removed from the diet.

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EXPLANATION OF PLATES

PLATE 12

FIG. 1. The kidneys from experiment described in Table IV in which phosphate in various forms was fed for 44 days. The upper row is of the control animals (Experiment 1a); from above down each row shows the effect of H_3PO_4 (Experiment 2); Na_3PO_4 (Experiment 5); $\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$ (Experiment 6); same mixture $\times 1.5$ (Experiment 7); same mixture $\times 2.0$ (Experiment 8); and $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$ (Experiment 9).

PLATE 13

FIG. 2. The typical lesion after 6 days of a diet containing a mixture of 3.19 per cent Na_2HPO_4 and 2.7 per cent NaH_2PO_4 . Above, the cortex with its convoluted tubules and glomeruli is unaffected. The lower two-thirds of the figure is occupied by the outer stripe of the outer zone. The pattern of the tubules is completely disorganized by the necrosis, atypical regeneration and calcification. Hematoxylin and eosin stain. $\times 150$.

FIG. 3. The initial lesion from the kidney of a rat fed 48 hours on a diet containing 10 per cent Na_2HPO_4 . The outer stripe of the outer zone of the medulla. Most of the terminal portions of the proximal convoluted tubule are normal, but extending down through the center of the figure is the end-piece of one whose epithelium is entirely necrotic. Hematoxylin and eosin stain. $\times 350$.

PLATE 14

FIG. 4. The outer stripe of the outer zone of the medulla from the kidney of a rat fed 15 days on a diet containing 10 per cent Na_2HPO_4 . Exuberant and atypical regeneration of the tubular epithelium distorts the tubule pattern. Many are transformed to solid structures. Giant cell-like masses are seen in several places. The two open areas on each side of the figure are spaces that were filled with calcareous material which has broken out of the tissue in sectioning. Hematoxylin and eosin stain. $\times 350$.

FIG. 5. The cortex from a kidney of a rat fed 44 days on a diet containing NaH_2PO_4 and Na_2HPO_4 , (Experiment 6 of Table IV and Fig. 1). Above the glomeruli and periglomerular proximal convolutions are fairly well preserved. Below and to the right fibrosis and round cell infiltration about collapsed distal convolutions are seen. Hematoxylin and eosin stain. $\times 150$.

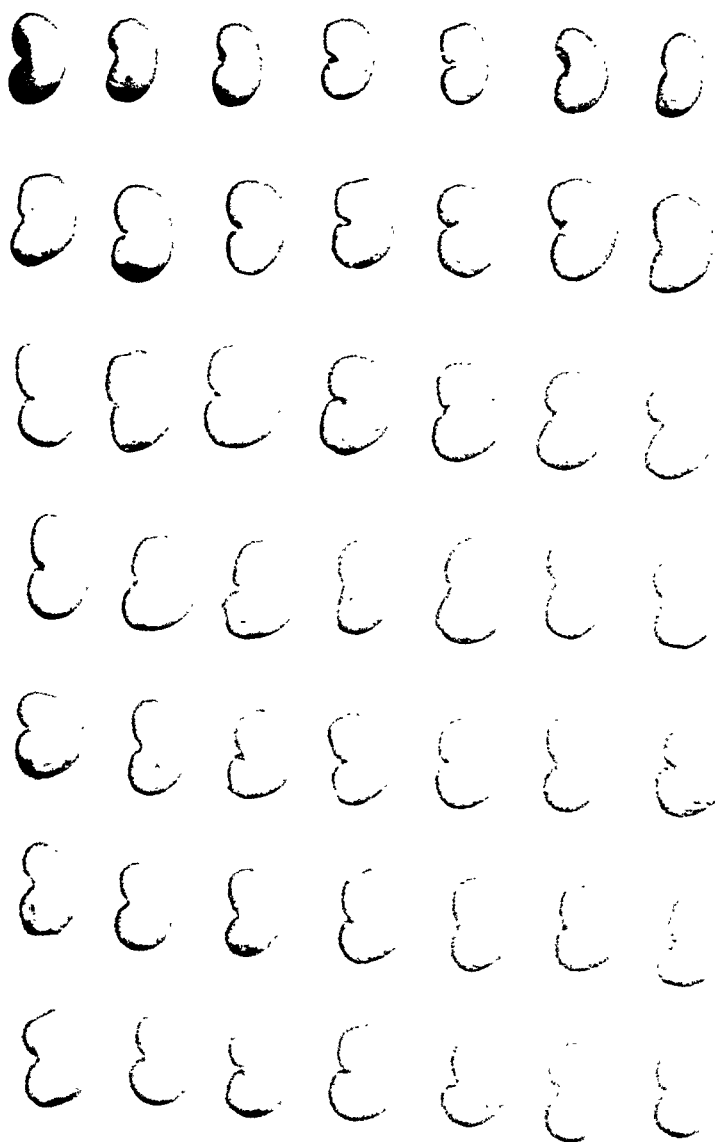


Fig. 1

Fig. 2

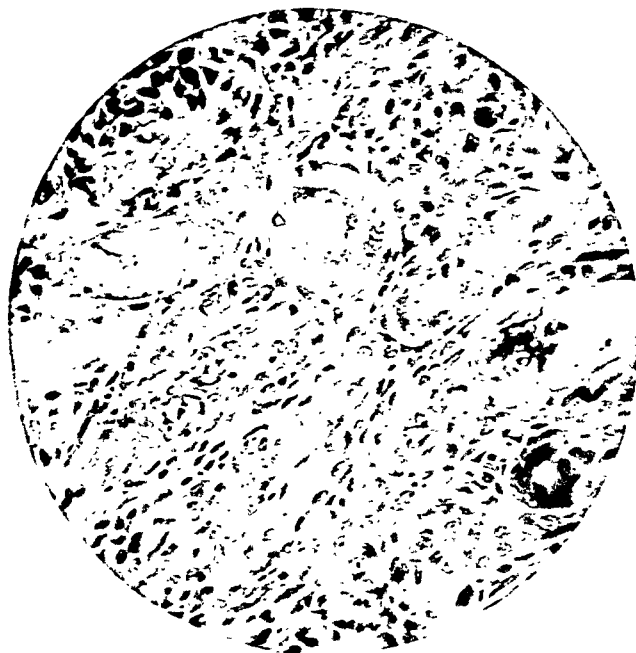


FIG. 4



FIG. 5

THE SEROLOGICAL DIFFERENTIATION OF PATHOGENIC AND NON-PATHOGENIC STRAINS OF HEMOLYTIC STREPTOCOCCI FROM PARTURIENT WOMEN*

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Hemolytic streptococci may cause several different clinical types of infection or, on the other hand, may be merely saprophytic and harmless to the human host. Acute tonsillitis and scarlatina, for example, are due to hemolytic streptococci, but a proportion of normal individuals habitually carry hemolytic streptococci in the throat without any apparent harm, and so far as it is known, without infecting other people. The organisms from both infected throats and healthy carriers are indistinguishable by any known test, and why one group of persons should be sick and the other go unscathed has never been satisfactorily explained.

The same phenomenon occurs in the human birth canal before and after delivery; most severe infections of the uterus which follow childbirth are due to hemolytic streptococci (1, 2); but these organisms may be present in the birth canal before and after delivery without the host showing any sign associated with active infection (3, 4).

Taylor and Wright (3) were unable to distinguish, by any of the tests at their disposal, between the organisms isolated from the vagina of febrile and afebrile cases. Hare (5) has, however, advanced evidence showing that most of the saprophytic¹ strains can be differentiated from the infecting strains in being sus-

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¹ The terms "saprophytic" and "non-infective" are used throughout this paper to imply failure to induce disease in the human host, although it is known that some, at least, of the strains which are saprophytic in the human vagina are nevertheless highly pathogenic for certain other animals.

ceptible to the bactericidal action of normal human blood. Hare and Colebrook (6) have also shown that a large proportion of the saprophytic strains in the vagina can be differentiated from infecting strains, since many of the former are, apparently, members of the group of hemolytic streptococci which causes a large proportion of cases of bovine mastitis. These workers employed biochemical tests to differentiate their strains; but Lancefield (7) has shown that it is possible to distinguish mastitis and other animal-infecting strains from strains from human infections by precipitin tests. She described four groups, each characterized by its own precipitinogen, or C substance. Group A consisted almost entirely of organisms from human infections (these strains giving a pH of 5.0 to 5.4 in 1 per cent glucose broth and failing to hydrolyze sodium hippurate); Group B, of organisms mainly from bovine mastitis (hydrolyzing sodium hippurate and giving a low pH in 1 per cent glucose broth); Group C, of organisms from a variety of animal sources (failing to hydrolyze sodium hippurate, giving an intermediate pH in 1 per cent glucose broth, and usually differing from the former groups in fermenting sorbitol and not trehalose); and Group D, a group of eight strains derived from cheese (giving a low pH in 1 per cent glucose broth, having little or no action on sodium hippurate, fermenting both sorbitol and trehalose, and reducing methylene blue milk).²

As the majority of the saprophytic strains isolated by Hare and Colebrook (6) gave the biochemical reactions of members of one or other of the serological Groups B and D described by Lancefield, the whole series of their strains were re-examined in order to determine whether:—(a) the strains which they had differentiated by biochemical reactions could also be differentiated by a serological test (precipitin reaction); (b) certain strains from uninfected cases which could not be differentiated biochemically from infecting strains were or were not members of Lancefield's Group A when tested serologically. The following experiments were therefore designed in order to elucidate these points.

Source of Strains

Series 1. From Cases of Definite Puerperal Uterine Infection.—These strains, 46 in number, were obtained from clinically typical cases of puerperal infection, many of which were fatal, in the Isolation Block of Queen Charlotte's Hospital, London. These women came from all parts of London after infection had developed. Only one of them had been delivered in Queen Charlotte's Maternity Hospital, and none came from the District.

² Lancefield also described a fifth Group E, of which she had but three representatives.

Series 2. From Women after Delivery Who Were either Uninfected or Who Had Only a Slight Increase of Temperature during the Puerperium.—Vaginal swabs were taken on the 3rd or 4th day after delivery from the majority of women delivered in Queen Charlotte's Maternity Hospital, over a period of 9 months. Cultures were made irrespective of whether the patient had fever or other signs of an abnormal puerperium. Streptococci giving areas of hemolysis on blood agar plates were isolated from 85 of the 837 women so examined. One of the 85 women was definitely infected, and she died 8 days after delivery from peritonitis and septicemia. The strain from this woman is included in the preceding series. 65 of the women had no elevation of temperature during the puerperium (a temperature of 100°F. on only one occasion being adjudged sufficient evidence to exclude a woman from this category) and it was therefore assumed that despite the presence of the organisms in the birth canal, no active infection was present. The remaining 18 women in this series had some fever during the puerperium but not enough to warrant their definite diagnosis as cases of puerperal uterine infection. Moreover, some of the women had extragenital infections of sufficient severity to cause the elevation of temperature which was observed.

Series 3. From Pregnant Women Immediately before Delivery.—Swabs were taken from the upper part of the vagina of every woman on the District of Queen Charlotte's Hospital immediately before delivery and before examinations of any sort had been made. In thirteen of the 855 women so examined, hemolytic streptococci were found to be present.² Twelve of these women with positive cultures subsequently had an afebrile puerperium, although hemolytic streptococci were still present after delivery in the majority of them. The other carrier had some fever during the puerperium, but it was slight and there were no other signs of puerperal uterine infection. Of the remaining 842 women, none subsequently became infected with hemolytic streptococci. For these strains we are indebted to Dr. R. M. Fry.

Isolation of the Strains

The original cultures were made on horse blood agar plates and incubated anaerobically in a McIntosh and Fildes jar for 16 hours (6, 8). Any hemolytic colonies were fished and the cultures purified by plating. The strains were stored in Robertson's meat medium; and subcultures in 5 per cent horse serum broth were inoculated into the various media employed for the tests.

² Only organisms able to form soluble hemolysin were studied from this series of patients. For this reason pseudohemolytic streptococci (strains giving marked hemolysis on blood plates but unable to form soluble hemolysin in the routine tests employed, *vide infra*) although known to be present, were not collected and studied.

Methods

1. *Immune Sera*.—Immune sera were prepared by the injection of rabbits with formalinized cultures as previously described (7).

2. *Extracts*.—Extracts were made by heating the organisms with hydrochloric acid as in the earlier experiments (7).

3. *Precipitin Tests*.—Most of the serological tests were made in America by one of us, according to methods already fully described, with strains sent from England. The remaining tests were carried out in England by the other author with sera sent from America; in the latter tests capillary pipettes as used by Day (9) were employed with the same relative proportions of serum and extracts as were used by Lancefield but in much smaller volume. The actual technique was as follows:

Two volumes (about 5 c.mm. per volume) of undiluted extract or extract diluted one-fourth with saline, were mixed with one volume of undiluted serum on a paraffined slide. The mixture was aspirated into a capillary pipette, which was then sealed, incubated at 37°C. for 2 hours, and placed in the ice chest overnight. The precipitates were easily visible with a hand lens. Control tests with larger volumes of serum and extract were found to give comparable results.

4. *Biochemical Reactions*.—These consisted of the following tests which were performed and described previously by Hare and Colebrook (6):—(a) formation of soluble hemolysin; (b) final pH after growth for 4 days in 1 per cent glucose broth; (c) hydrolysis of sodium hippurate; (d) growth on 40 per cent bile blood agar; (e) digestion of human fibrin; (f) fermentation of sorbitol, trehalose, lactose, mannite, and salicin.

RESULTS

Series 1. Strains from Definite Puerperal Infections of the Uterus.—In Table I are given the serological and biochemical tests carried out with 46 strains from definite cases of puerperal uterine infection. All except one strain were classified in Group A as a result of the precipitin tests, which showed a sharp and unmistakable differentiation of the various groups. The biochemical reactions of these hemolytic streptococci were, in the majority of instances, of the type usually associated with strains from human infections although some exceptional strains were encountered. It is also noteworthy that all the Group A strains were able to digest human fibrin (10, 11). The one exception to the serological similarity among these strains from infected cases was a Group G strain isolated from the blood of a fatal case of septicemia. The patient was also suffering from an overwhelming infection with *Staphylococcus aureus*, and it is possible that

the hemolytic streptococci were secondary invaders. It was also noted that biochemically this strain was very atypical.

Series 2. Strains Isolated after Delivery from Women Who Were either Uninfected or Who Had Only a Slight Increase of Temperature during the Puerperium.—The serological and biochemical reactions of the 66 strains isolated on the 3rd or 4th day of the puerperium from the 65 women who had no elevation of temperature at any time during the puerperium (members of two groups being isolated from one patient) are given in Table II.

TABLE I

Series 1: Strains from Severe Cases of Hemolytic Streptococcal Infection of the Uterus

No. of strains	Precipitation with sera from Group						Formation of soluble hemolysin	pH in 1 per cent glucose broth	Hydrolysis of sodium hippurate	Growth on 40 per cent bile agar	Digestion of human fibrin	Fermentation of				
	A	B	C	D	F	G						Sorbitol	Trehalose	Lactose	Mannite	Salicin
37	+	-	-	-	-	-	++++	5.0-5.4	-	-	+	-	+	+	-	+
1	+	-	-	-	-	-	++++	4.9	-	-	+	-	+	+	-	+
3	+	-	-	-	-	-	++++	5.2-5.4	-	-	+	+	+	+	+	+
1	+	-	-	-	-	-	++++	5.0	-	+	+	+	+	+	+	+
1	+	-	-	-	-	-	++++	5.0	-	±	+	-	+	+	-	+
1	+	-	-	-	-	-	++++	5.4	-	-	+	-	+	±	+	+
1	+	-	-	-	-	-	++++	5.4	-	-	+	-	-	+	-	+
1	-	-	-	-	-	+	+	5.0	-	+	-	-	+	+	-	+
Totals...46	45	0	0	0	0	1										

* Patient had *Staphylococcus aureus* septicemia.

Only one Group A strain was isolated from these afebrile patients, and this was the only instance in this study in which a Group A strain was found in the vagina unassociated with disease. All of the other strains from afebrile patients post partum, fell into other groups or were unclassified; the 26 members of Group B were, with one exception, also distinguishable from other groups by their biochemical reactions, as were also the 26 members of Group D. The five members of Group C, as well as the two Group F, the three Group G, and the three unclassified strains, were somewhat variable in their biochemical reactions but could be clearly differentiated from the pathogenic strains by means of the precipitin test.

In Table III are given the reactions of the 18 strains from the 18 women in this series who had some degree of fever during the puerperium. It must be stressed that there was much doubt with most of these women whether this pyrexia was really due to a uterine infection. Certainly none of the cases could be compared with those enumerated in Table I in point of severity. The relevant clinical

TABLE II

Series 2: Strains Isolated Post Partum from Women with an Afebrile Puerperium

No. of strains	Precipitation with sera from Group						Formation of soluble hemolysin	pH in 1 per cent glucose broth	Hydrolysis of sodium hippurate	Growth on 40 per cent bile agar	Digestion of human fibrin	Fermentation of				
	A	B	C	D	F	G						Sorbitol	Trehalose	Lactose	Mannite	Saltin
1	+	-	-	-	-	-	++++	5.4	-	-	+	-	+	+	-	+
25	-	+	-	-	-	-	+ to +++	4.2-4.9	+	+	-	-	+	+	-	+
1	-	+	-	-	-	-	+	4.6	-	+	-	+	+	-	-	-
2	-	-	+	-	-	-	++++	5.4	-	-	+	-	+	+	-	+
1	-	-	+	-	-	-	++++	5.0	-	+	+	-	+	+	-	+
1	-	-	+	-	-	-	++++	5.4	-	-	+	-	+	+	-	-
1	-	-	+	-	-	-	++++	5.2	-	-	+	-	+	+	-	-
26	-	-	-	+	-	-	-	4.2-4.8	-	+	-	+	+	+	+	+
1	-	-	-	-	+	-	+	5.0	-	-	-	-	-	+	-	+
1	-	-	-	-	+	-	+	5.0	-	-	-	-	+	+	-	+
3	-	-	-	-	-	+	++++	5.2-5.4	-	-	+	-	+	+	-	+
1*	-	-	-	-	-	-	++++	5.4	-	-	-	-	+	+	-	+
1*	-	-	-	-	-	-	+++	4.8	-	-	-	-	+	+	-	+
1	-	-	-	-	-	-	-	4.8	-	+	-	+	+	+	+	+
Totals. .66†	1	26	5	26	2	3										

* These two strains was also tested with sera of Group E and Strain K 130. Negative reactions were obtained.

† 3 unclassified strains are included in this total.

details of these "minor infections" are also given in Table III. It is evident that none of the 18 strains fell into Group A. One strain was in Group C, one in Group G, and one unclassified; seven strains were members of the bovine mastitis Group B, and eight were "pseudo-hemolytic streptococci," members of Group D. It would seem, therefore, that some of the minor infections which accompany childbirth may possibly be due to organisms of other groups than Group A.

TABLE III
 Series 2: Strains Isolated from Minor Infections

Name	Temperature	Serological group	Formation of soluble hemolysin	pH in 1 per cent glucose broth	Hydrolysis of sodium hippurate	Growth on 40 per cent bile agar	Digestion of human fibrin	Fermentation of				
								Sorbitol	Trehalose	Lactose	Mannite	Saline
W-y	101°F. on 2nd day	C	++++	5.0	—	—	—	—	—	—	—	—
M-d	100.6°F. on 4th and 5th days	G	++++	5.2	—	—	+	—	+	+	—	+
B-y	99.4°F. on 3rd, 100.4° on 4th, 99.4° on 5th days	B	++	4.4	+	+	—	—	+	+	—	+
S-s	100°F. on 2nd, 4th, and 6th days	B	+++	4.6	+	+	—	—	+	+	—	+
T-n	101.4°F. on 5th, 100.2° on 6th days	B	+++	4.5	+	+	—	—	+	+	—	+
M-n	99–100°F. for 7 days	B	+	4.7	+	+	—	—	+	+	—	+
C-c	103°F. on 4th day (cold ?)	B	+	4.6	+	+	—	—	+	+	—	+
C-k	100°F. on 1st day	B	+	4.5	+	+	—	—	+	+	—	+
W-f	100°F. on 2nd, 101.4° on 6th, and 102.8° on 8th days (influenza ?)	B	+	4.8	+	+	—	—	+	+	—	+
P-c	101.4°F. on 7th, 101.8° on 8th days (probably due to breast abscess)	D	±	4.6	±	+	—	+	+	+	+	+
S-d	102°F. on 1st day	D	—	4.5	—	+	—	+	+	+	+	+
B-m	101.6°F. on 8th day	D	—	4.5	±	+	—	+	+	+	+	+
M-n	102°F. on 1st day	D	—	4.5	±	+	—	+	+	+	+	+
C-r	101.6°F. on 2nd, 100.4° on 3rd, 99.4° on 5th days	D	—	4.7	±	+	—	+	+	+	+	+
F-g	100°F. ± for 7 days	D	—	4.6	—	+	—	+	+	+	+	+
B-t	100–102°F. for 7 days	D	—	4.6	—	+	—	+	+	+	+	+
D-g	100°F. on 5th, 100.4° on 8th days (pyelitis)	D	—	4.5	—	+	—	+	+	+	+	+
L-n	100–101°F. for 4 days	U*	+++	5.4	—	—	+	—	+	+	—	+

* Indicates unclassified strain.

Thus, in the whole series of 837 women examined after delivery in Queen Charlotte's Maternity Hospital, organisms giving areas of hemolysis on blood agar were isolated from (a) one undoubted case of puerperal infection, (b) 18 cases of minor infection, and (c) 65 women who had an absolutely afebrile puerperium. The strain from the definitely infected case fell into Group A, and, with the exception of one Group A strain from an afebrile patient, none of the strains from the afebrile patients or those with minor infection were identified as members of Group A.

TABLE IV

*Series 3: Strains Isolated Ante Partum from Women Whose Subsequent Puerperium Was Afebrile**

No. of strains	Precipitation with sera from Group						Formation of soluble hemolysin	pH in 1 per cent glucose broth	Hydrolysis of sodium hippurate	Growth on 40 per cent bile agar	Digestion of human fibrin	Fermentation of				
	A	B	C	D	F	G						Sorbitol	Trehalose	Lactose	Mannite	Salicin
5	—	+	—	—	—	—	+ to ++	4.6-4.8	+	+	—	—	+	+	—	+
1	—	—	+	—	—	—	++++	5.4	—	—	+	—	+	+	—	+
3	—	—	—	—	—	+	++++	5.2-5.4	—	—	+	—	+	+	—	+
2	—	—	—	—	—	+	++	4.6	—	+	—	—	+	+	—	+
Total...11	0	5	1	0	0	5										

* Group B strains were isolated both before and after delivery from one additional patient who had a minor degree of pyrexia during the puerperium.

Series 3. Strains from Cultures Taken Immediately before Delivery.—The reactions of eleven of the twelve strains (one strain was lost) isolated before delivery from women whose subsequent puerperium was afebrile are given in Table IV. It will be seen that none of these strains, saprophytic in the vagina, fell into Group A. The remaining patient had slight pyrexia during the puerperium and the strains isolated before and after delivery were both found to belong to Group B by serological and biochemical tests.

It is thus clear that the vast majority of strains from severe puerperal infections due to hemolytic streptococci are members of Group

A, and that most of those which are saprophytic in the birth canal either before or after delivery, as well as those strains isolated from women with minor degrees of fever, do not belong to Group A. Hare and Colebrook (6) were able to differentiate by means of biochemical tests the majority of these saprophytic strains (those placed in Groups B and D in this paper) from the strains isolated from infections. The present work confirms their findings in this respect. However, the serological tests reported here show that some non-infective strains which Hare and Colebrook found biochemically similar to those from infections but which they were unable to differentiate from infecting strains can, nevertheless, be differentiated by their group precipitinogen (those placed in Groups C, F, and G in this paper). The information available on these points may be summarized in the form of a table (Table V).

Comments on the Serological and Biochemical Reactions of Strains from the Different Groups

Facts relevant to the serological groups into which these organisms have been classified, and a description of the two new serological groups found in this series of hemolytic streptococci are given below:—

Group A.—The Group A strains, derived from human puerperal infections of a severe type, all form a C substance which gives precipitates with the appropriate Group A anti-C sera. Their biochemical reactions, with a few exceptions, are those of *Streptococcus pyogenes*.

Group B.—Organisms placed in this group, by reason of their serological reactions, give the biochemical reactions of certain bovine mastitis organisms; that is to say, they hydrolyze sodium hippurate, give a low pH in 1 per cent glucose broth, grow on 40 per cent bile agar, and form only small amounts of soluble hemolysin.

The Group B strains in the present series of hemolytic streptococci isolated from women were classified into types in order to determine whether they belonged to the same serological types encountered among the Group B strains derived from cows (12). The majority of the strains (34 out of 39) were tested and all except one strain fell into one or other of the specific types originally differentiated among strains of bovine origin. In all, 39 strains of this group have been isolated from the human vagina before or after delivery. Plummer (13) has also found organisms of this group in normal human throats. One of us has confirmed this. There can be little doubt, therefore, that Group B organisms can

exist as saprophytes in the human throat and vagina. But in view of the evidence set forth in Table III it would seem that a proportion of low grade infections may be due to organisms of this group.

Group C.—Group C (7) contains strains which are similar biochemically to pathogenic human strains but which are distinguishable by the specific anti-C

TABLE V
Serological Grouping of All Strains Examined

Time of culture	No. of women examined	Clinical condition of patient	No. of strains isolated	No. of strains in each serological group					
				A	B	C	D	F	G
Before delivery (Queen Charlotte's Hospital District)	855	Afebrile puerperium Minor infection	11* 1		5 1	1	†		5
After delivery (Queen Charlotte's Hospital)	837	Afebrile puerperium Minor infection Definite infection	66† 18 1	1 7 1	26 1	5 1	26 8	2 1	3 1
After delivery (Queen Charlotte's Hospital Isolation Block)§	45	Definite puerperal infection	45	44				1	
Totals.....			142	46	39	7	34	2	10

* One more strain was isolated but was lost.

† Only organisms which formed soluble hemolysin were studied in this group of patients. All pseudohemolytic streptococci studied from other patients have fallen into Group D.

‡ Members of two groups (B and G) were isolated from one patient.

§ The members of this group were not derived from the groups studied in Queen Charlotte's Hospital and on the District. These patients were brought to the Isolation Block, which is entirely separate from the Maternity Hospital proper, from various parts of London at a time when they were already ill.

|| Also accompanying overwhelming *Staphylococcus aureus* infection.

reaction. The strains originally studied were all derived from animal sources other than man, gave marked hemolysis on blood agar plates, and formed soluble hemolysin. They usually differed from *S. pyogenes* of human origin in fermenting sorbitol and not trehalose, although four strains among the 49 examined by Lancefield (7) fermented neither of these substances. Other points of difference were in the attainment of a final pH in 1 per cent glucose broth inter-

mediate between that of Group A strains and that of Group B strains, and in the susceptibility of Group C strains to lysis by bacteriophage. Edwards (14) and also Ogura (15) have described hemolytic streptococci having similar characteristics, all derived from animals.⁴ Edwards has also shown that the strains he studied can be differentiated from human strains by precipitin tests, with the carbohydrate specific for Group C. In the present study, seven strains have been encountered, giving group precipitates with Group C antisera. All except one were saprophytic, and there is much doubt whether the remaining strain was really infecting the host. All these seven strains fermented trehalose and not sorbitol. In this respect they differed from the Group C strains from animal sources described by Lancefield, but were similar to the Group C strains recently described by Edwards and designated by him in conformity with Ogura's nomenclature, as Types B 1 and B 2. The seven Group C strains described in the present paper all ferment lactose in addition to trehalose, and would on this basis belong to Edwards' Type B 2, of which he described one strain and Ogura three. All seven were found capable of digesting human fibrin.

It may be mentioned in passing that one of us has also obtained from animal sources Group C strains which ferment trehalose but not sorbitol or lactose. One was from the throat of a normal dog and three were from the throats of *cynomolgus* monkeys. The other author has encountered sixteen Group C strains in the throats of normal human beings all of which fermented trehalose and lactose but not sorbitol.

It is of interest that the fermentation of salicin is somewhat variable among strains placed in this group. A definite proportion of the strains fail to ferment this substance.

Group D.—The eight strains of this group described by Lancefield (7) were all derived from cheese. Of the present series of strains, 34 were found to belong to this group, and their biochemical reactions were in all important respects similar to those originally studied by Lancefield. These 34 strains were described by Hare and Colebrook (6) as "pseudo-hemolytic streptococci" because they differed from organisms placed in the other groups in not forming soluble hemolysin, when tested by the usual methods,⁵ although giving complete and wide areas of hemolysis on blood agar plates. They also regularly ferment mannite and sorbitol as well as trehalose, lactose and salicin, and give a low pH in 1 per cent

⁴ It is probable that the S 21 series of Minett and Stableforth (16) are similar organisms.

⁵ The actual method consisted in the addition of one volume of a 12 to 16 hour culture in 20 per cent horse serum broth to one volume of 5 per cent horse red cells in saline and incubating the mixture at 37°C. for 2 hours. Todd (17) has since shown that by employing a highly buffered broth and a relatively lower temperature (30°C.) for the incubation of the cultures, Group D organisms do form sufficient soluble hemolysin to give a positive test when the cultures are added to red cells in saline.

glucose broth but have little or no action on sodium hippurate. Similar organisms have been described by Taylor and Wright (3) as occurring in the vagina, and by Weatherall and Dible (18) in the feces. One of us has confirmed this latter finding. These pseudohemolytic streptococci resemble the group of enterococci or *S. faecalis* more than *S. pyogenes* because they are characteristically lanceolate, grow in moist luxuriant colonies, and a proportion resist heat at 60°C. for 30 minutes. It is improbable that organisms of this group are responsible for severe infections, although one case of endocarditis due to them has been encountered.⁶

Group F.—This is a new group not hitherto described. The original members (four strains) were obtained from Dr. Perrin H. Long. Their morphology was originally described in abstract by Bliss and Long (20) and in more detail recently (21). Their chief characteristics are their slow and difficult growth, and the formation of minute transparent colonies with a relatively wide area of hemolysis. The original strains of Bliss and Long were obtained rarely from the throats of normal individuals and more often from patients with infections of the upper respiratory tract. In addition to the two Group F strains observed in the present series, others have been obtained from the tonsils of one patient and from the chest fluid of another patient in the Hospital of The Rockefeller Institute. The significance of these organisms in human infections is at present uncertain, although the two strains encountered in this study did not give rise to infection.

Group G.—This is another group not hitherto described. The immune sera used for its differentiation were made by the injection of rabbits with one of the strains studied here. The ten members of the group described in this communication resemble the members of Group A very closely in their biochemical reactions, including the ability to digest human fibrin, a property which seven of the ten strains possessed. Two additional saprophytic strains from the vagina and six strains from normal human throats have also been encountered, as well as one strain from a case of otitis media in a dog and one from a fatal case of pneumonia in a monkey. Four strains from the throats of normal monkeys have also been found.

DISCUSSION

The researches described in this paper are, in effect, an attempt to differentiate those hemolytic streptococci which are likely to do harm during childbirth from those which either do not or cannot do so.

⁶ It is improbable that the pseudohemolytic streptococci from the respiratory tract described by Cumming (19) are the same organisms. None of his strains fermented mannite, whereas all the Group D strains do so. After four subcultures none of the strains studied by Cumming showed hemolysis on rabbit blood agar plates although they were hemolytic on human blood agar. Furthermore, his strains were indistinguishable, microscopically and colonially, from *S. pyogenes*; Group D strains can, with a little practice be readily distinguished.

The precipitin tests show that the vast majority of strains from definite infections of the uterus are members of Group A. Lancefield (7) has also described a group of 21 strains mainly from infections of the human respiratory tract which gave precipitates with Group A sera. Their biochemical reactions were similar to those of the majority of the infective uterine strains described in this paper; that is, both groups failed to hydrolyze sodium hippurate or to grow on 40 per cent bile agar, but attained a low pH in 1 per cent glucose broth, digested human fibrin, and fermented trehalose, lactose, and salicin and, very occasionally, sorbitol and mannite. Although it is possible that other groups may later be found implicated as producers of human disease, as suggested particularly by the recent work of Long, Bliss, and Walcott (22), it appears at present that Group A strains are the hemolytic streptococci most likely to cause serious human infection.

The vast majority of hemolytic streptococci from the birth canal which do not bring about active infections are not members of this group. Most of them fall either into Group B or D; the former being identical with certain strains causing bovine mastitis, and the latter resembling *S. faecalis* more than *S. pyogenes*. These two groups were differentiated by Hare and Colebrook (6) by means of biochemical tests, and this differentiation has been here confirmed serologically. The remaining non-infective strains fell into Group C, F, or G, or were unclassified. In their biochemical reactions they resemble Group A, and for this reason Hare and Colebrook were unable to differentiate them from the infective strains. But that they differ immunologically from Group A strains can hardly be doubted in view of the results of the present study. It would therefore appear that the differentiation, by a comparatively easy precipitin test, of hemolytic streptococci which are potentially infective from those which are harmless to man is entirely feasible.

It seems highly probable that the human nasopharynx is the main reservoir of Group A strains in nature. Because of this, and because of the great rarity of Group A hemolytic streptococci in the normal vagina, *ante partum*, there can be little doubt that the vast majority of puerperal hemolytic streptococcal infections are due to inoculation from some other source than the patient's genital tract and prob-

ably arise from the above mentioned reservoir in the patient or attendants. Thus, indirectly, this work confirms that of Smith (23) and of Paine (24), who showed that the organisms in the majority of hemolytic streptococcal infections of the uterus could be identified by agglutinin absorption as the same as those present in the nose or throat of one or other of the attendants at the time of delivery.

The correlation between puerperal infection and the presence of Group A hemolytic streptococci in the vagina, on the one hand, and the corresponding lack of serious infection though hemolytic streptococci of other serological groups are often present, is set forth in the following summary.

TABLE VI
Summary of Results with Reference to Group A Hemolytic Streptococci

Source of cultures	No. of cases with hemolytic streptococci	No. of Group A strains isolated	Type of infection
Cases admitted to Isolation Block after delivery: 45	45	44*	All severe puerperal infection
Women delivered in Hospital (cultures taken post partum): 837	85	1 0 1	1 fatal puerperal infection 18 minor infections 65 afebrile cases
Women delivered on District (cultures taken ante partum): 855	13	0	1 minor infection 12 afebrile cases

* A Group G strain, together with a *Staphylococcus aureus*, was isolated from the remaining case.

It is, therefore, evident that hemolytic streptococci may be harbored in the birth canal either before or after delivery, without causing disease, provided they belong to serological groups other than Group A. Group A hemolytic streptococci, on the contrary, are usually absent from the vagina ante partum or are exceedingly rare, as shown by the failure to find organisms of this group in cultures taken before delivery from the present series of patients. However, Group A hemolytic streptococci if present in the vagina post partum almost always give rise to serious puerperal infection. According to these results, therefore, Group A strains are probably the only hemolytic

streptococci capable of causing definite puerperal infection in the human species, and such infection almost invariably occurs if Group A hemolytic streptococci are present in the vagina.

CONCLUSION

1. The majority of strains of hemolytic streptococci from puerperal infections of the uterus were identified serologically as members of the Group A described by Lancefield.
2. The majority of strains isolated from the birth canal of women whose puerperium was afebrile were not members of Group A.
3. The existence of two new serological groups of hemolytic streptococci, Groups F and G, is described.

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STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

III. THE EFFECT OF EXTRANASAL INJECTION ON THE GROWTH OF THE FOWL CORYZA BACILLUS

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Observations on the nature and etiology of an uncomplicated coryza of the domestic fowl were presented in earlier papers (1-3). Susceptible fowl injected intranasally with exudate or with pure cultures of a hemophilic bacillus isolated from exudate regularly showed an inflammation of the nasal mucosa. The hemophilic bacillus, which is closely related to if not identical with one earlier reported by de Blicke (4), is here referred to as the fowl coryza bacillus.

The present experiments were undertaken to determine whether or not the specific bacillus was able to multiply in loci outside the nasal passages and whether or not it was able to migrate from these loci to the upper respiratory tract. Attention was focused on this problem by the occasional appearance of pathological changes in the trachea and orbital tract of birds naturally or experimentally infected with coryza.

In the case of tracheal involvement the respiration was audible and characterized by a gurgling or rasping sound. There was a tendency for the affected bird to breathe with the mouth open, in some cases gasping for breath. These symptoms appeared to be referable to a partial occlusion of the trachea by exudate. Concerning the orbital tract, plates of exudate of either a rubbery or cheesy consistency were present beneath the eyelids. Occasionally the exudate was sufficient to cause an outward bulging of the lid. In some cases there was a thin watery discharge which was generally accompanied by a foamy film between the lids.

EXPERIMENTAL

Intratracheal Injection

A series of 10 susceptible birds were injected intratracheally with infective exudate and another series of 6 with pure cultures of the fowl coryza bacillus.

The exudate employed in this and the subsequent experiments was removed from the upper respiratory tract of birds experimentally infected with a coryza of rapid onset and long duration. In the case of the birds which were injected intratracheally, exudate from the trachea of a few birds which had shown spontaneous involvement of that locus was also used. This type of coryza has been maintained for over a year by bird to bird transfer. Recently isolated cultures of the fowl coryza bacillus were employed, new cultures being substituted for the old as the work progressed. The organism was cultivated for 1 to 2 days at 37°C. in fluid chicken serum at the base of slanted nutrient agar. Unless otherwise indicated approximately 0.5 cc. amounts of exudate, diluted with a little bouillon, and of

TABLE I
Intratracheal Injection of Exudate

Source of exudate	Bird No.	Symptoms of tracheal involvement	Discharge from nares	Length of time to autopsy	Inflammation of trachea at autopsy	Isolation of coryza bacilli from trachea
				days		
Nasal passages	1	—	+ 8th day	8	—	
	2	—	—	8	—	
	3	—	—	6	—	
Trachea	4	—	—	14	—	
	5	—	+ 3rd day	8	—	—
	6	+ 4th day	+ 2nd day	5	+	+
	↓					
	7	?	+ 4th day	6	+	+
	↓					
	8	+ 4th day	+ 5th day	6	+	+
	↓					
	9	+ 1st day	+ 2nd day	3	+	+
	↓					
	10	—	—	18	—	—

undiluted culture fluid were employed. The injected birds which were generally between 2 and 3 months of age, were maintained under strict quarantine, and were examined daily during the period of observation.

The results of the experiment on the intratracheal injection of exudate are summarized in Table I. The 3 birds which had received exudate from the nasal passages showed neither symptoms of respiratory difficulty during life nor an inflammation of the trachea at autopsy. One of the birds developed a coryza on the 8th day.

Two of the birds which had received tracheal exudate likewise showed no involvement of the trachea. The respiration of the third

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fowl was distinctly audible and rasping on the 4th day after injection. At autopsy, on the 5th day, the trachea was congested and coated with a layer of mucocellular exudate which contained epithelial cells, polynuclear leucocytes, red blood cells, and Gram-negative bacilli. Tracheal involvement was subsequently initiated in 3 susceptible fowl by direct bird to bird passage of tracheal exudate. These 3 birds all showed a catarrhal inflammation of the trachea together with petechial hemorrhages. Symptoms of respiratory difficulty were apparent in 2 and questionable in the third. A fourth serial passage was attended by negative findings, the injected bird showing no indication of tracheal involvement.

Five of the birds which had received tracheal exudate developed a coryza, at intervals varying from 2 to 5 days after injection. One bird showed, in addition, a watery discharge from both eyes. Cultures of the fowl coryza bacillus were obtained only from the tracheas which contained exudate.

Only one of the 6 birds which were injected with the fowl coryza bacillus in pure culture showed an involvement of the trachea. The respiration of this bird was audible, with a gurgling sound, on the 7th day after injection. At autopsy, on the same day, the trachea showed no changes save for a slight increase in mucus and culturally was sterile. Tracheal scrapings from this bird, injected intratracheally into a susceptible fowl, produced a gurgling respiration on the 3rd day and, at autopsy, on the same day, a marked catarrhal inflammation of the trachea with petechial hemorrhages. The fowl coryza bacillus was isolated from the tracheal exudate. A second serial passage of tracheal exudate was not attended by an involvement of the trachea in the injected bird.

Two of the birds which had been injected with cultures and the bird which had received the second serial passage developed a coryza on the 4th, 1st, and 3rd day, respectively.

Intraorbital Injection

Two groups of 3 birds each, were injected intraorbitally with exudate and culture fluid, respectively. The injections were made bilaterally, a few drops of the infective material being introduced under the lower eyelid.

The 3 birds which had received exudate showed no local involvement during the period of observation. Two were killed and autopsied

a week after injection and the third at the end of the 2nd week. Two of the birds developed a coryza after 6 and 7 days, respectively. Post-mortem examination revealed inflammatory manifestations only in the upper air passages of the 2 birds which had shown a nasal discharge.

The fowl coryza bacillus was not isolated from lacrimal duct cultures which were made on the 4th day after injection. A few colonies of the specific bacillus were obtained, however, from the lacrimal duct of one of the birds which developed a coryza.

The 3 birds which were injected with culture fluid likewise showed no indication of a local involvement, either during life or at autopsy. Two of these birds developed a coryza, in one case on the 3rd day after injection and in the other on the 4th day. These 2 fowl were kept under observation for a 10 day period after recovery from the coryza had occurred. The duration of the nasal discharge was 15 days in one bird and 8 days in the other. In neither case was there any visible involvement of the exterior portion of the orbital tract during this extended period.

Intra-aural Injection

Two groups of 3 birds were injected intra-aurally with exudate and culture fluid, respectively. A few drops of the inoculum were introduced into both tympanic cavities by way of the external auditory meatus.

. Of the group which received exudate, one bird showed a nasal discharge on the 7th day after injection. This bird was killed on the same day and at autopsy a mucopurulent exudate was present in the nasal cavities and in the right orbital sinus. A pure growth of the coryza bacillus was obtained from both loci. The aural tract was normal. The 2 remaining birds which were held for a period of 2 weeks showed no involvement of the organs of hearing or of the upper air passages.

One of the birds which was injected with culture fluid likewise showed a coryza, the nasal discharge appearing on the 6th day. This bird was killed on the 21st day of the discharge and autopsied. It may be noted that the coryza produced by the specific bacillus rarely persists for so long a period. The nasal cavities and sinuses contained a large volume of exudate from which the specific bacillus was not isolated. The tympanic cavities and auditory canals were normal. Two of the birds showed no inflammatory reaction in either the aural or upper respiratory tract.

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Intracloacal Injection

Three birds were each injected intracloacally with infective exudate and a similar number with culture fluid.

The birds in both groups remained normal throughout an observation period of 2 weeks. There was no indication, in any case, of an inflammatory involvement of either the lower intestinal or upper respiratory tract. The injected birds were held for susceptibility tests during an additional period of several weeks. Postmortem examination made at the conclusion of this test revealed no macroscopic changes in the intestinal tract or in the visceral organs.

On the 2nd day after injection sealed plates were streaked from the cloacal mucosa of 2 of the birds which had received culture fluid. The fowl coryza bacillus was not isolated in either case.

Intravenous Injection

Six birds were each injected intravenously, in the large wing vein, with 1.0 cc. of a 24 hour chicken serum culture of the fowl coryza bacillus. Because of the risk entailed by the injection of numerous secondary bacteria, including staphylococci and streptococci, exudate was not employed in this experiment nor in the two which followed. The injected birds were kept under observation for 4 to 18 days and then killed.

Two of the birds refused food during the first 2 days after injection and were noticeably inactive. They subsequently regained their normal vigor and remained healthy through the termination of the experiment. The 4 remaining birds showed no symptoms at any time during the period of observation. At autopsy, the nasal passages were normal in each case and no abnormalities were encountered elsewhere.

Cultures were made in each instance from the mucosa of the upper air passages, the spleen, and the heart's blood. They were uniformly negative for the fowl coryza bacillus.

Subcutaneous Injection

Seven birds were each injected under the skin of the upper and outer portion of the leg with 0.75 to 1.0 cc. of culture fluid.

All of the birds showed a local reaction consisting of a slight swelling and discoloration. The duration of this reaction varied in different

birds, in one instance being still noticeable on the 10th day after injection. In 4 of the birds which were killed on the 7th day it was no longer visible. There was no indication of a systemic involvement in any case. None of the birds showed a nasal discharge during the period of observation.

Postmortem examination revealed a tough yellowish plate of exudate beneath the skin at the site of injection. This local mass of exudate, measuring in one case 6.5 by 1.5 cm., was loosely adherent to the underlying tissue which was hemorrhagic and thickened. The skin over the involved area was also thickened and somewhat congested. The local exudative reaction was observed in birds killed as early as the 4th day and as late as the 10th day after injection. The abdominal organs, lung, and upper respiratory tract showed no visible pathological changes in any case.

The fowl coryza bacillus was isolated in only two instances from the local exudate. Cultures from the nasal mucosa and the spleen were uniformly negative.

Intraperitoneal Injection

Twelve birds were injected intraperitoneally with 1.0 cc. amounts of culture fluid. Seven individuals of this series were given a single injection and 5 were given 2 injections spaced 48 hours apart.

The results of this experiment are summarized in Table II. One bird, No. 12, showed a labored breathing in the morning of the 3rd day and was found dead the same afternoon. No visible pathological changes were found at autopsy. A second bird, No. 3, refused food for 2 days after injection but subsequently regained its normal state which continued during the period of observation. The remaining birds showed no symptoms suggestive of a systemic reaction.

A coryza occurred in a single case, Bird 5, which showed a nasal discharge on the 3rd day after injection. At autopsy, a mucopurulent exudate was present in the nasal cavities. Postmortem examination of the upper respiratory tract showed no inflammatory involvement in the other birds. Minor changes were found in the abdominal cavity of 4 birds, Nos. 2, 3, 8, and 11. In 2 cases a small amount of tenacious exudate was reflected as a membrane over a portion of the liver. In 2 other cases small encapsulated masses of exudate were present in the omental tissue. No visible lesions were encountered in the other birds.

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Cultures from the nasal passages were made in each instance. The fowl coryza bacillus was isolated, generally in small numbers, from the nasal mucosa of 7 of the birds. The specific bacillus was isolated in pure culture from the heart's blood of the single fatal case. Heart's blood cultures from the other birds were all negative. Positive spleen cultures were obtained in 2 instances only, Birds 2 and 5. Cultures were also made from the liver and omentum of the fowl which had shown pathological changes in these loci. The fowl coryza bacillus was

TABLE II
Intraperitoneal Injection of the Fowl Coryza Bacillus

No. of bird	Discharge from nares	Length of time to autopsy days	Isolation of the specific bacillus		
			Nasal passages	Spleen	Heart's blood
1	—	7	+	—	—
2	—	7	+	+	—
3	—	7	+	—	—
4	—	7	+	+	—
5	+ 3rd day	3	—	—	—
6	—	7	—	—	—
7	—	2	—	—	—
8*	—	9	—	—	—
9	—	9	+	—	—
10	—	9	—	—	—
11	—	3	—	—	+
12	—				

* The last 5 birds received 2 intraperitoneal injections.

isolated in only one instance, from the omentum of Bird 8. Three of the cultures which were obtained from the upper air passages were injected intranasally in susceptible fowl and found to be virulent.

DISCUSSION

In analyzing the preceding experimental findings it is essential that they be compared with the results of direct intranasal injection of exudate and the specific bacillus. In our experience a coryza invariably follows the injection of recently drawn exudate or virulent strains of the bacillus. With the dosage employed in routine testing,

the specific organism has never failed to develop in the upper air passages and has never failed to provoke an inflammatory reaction.

The implantation of the specific bacillus, whether in exudate or in pure culture, on other mucous surfaces as those of the trachea, orbital cavity, internal ear, and cloaca was attended by a local multiplication of the organism only in the case of the trachea. The trachea was also the only locus in which injection was followed by a visible inflammatory reaction. Development of the specific bacillus even when directly introduced in the trachea was not, however, a constant finding.

The experimental findings indicate, however, that a nasal carriage of the bacilli from the trachea, orbital cavity, and internal ear may be affected in the absence of a local multiplication and a coryza may ensue. These loci are in more or less direct communication with the upper air passages by way of the palatine cleft, the Eustachian tubes, and the lacrimal ducts, respectively. There was no evidence of a carriage of the bacilli from the cloaca.

The results of intravenous, subcutaneous, and intraperitoneal injection, by which the bacilli were brought in contact with surfaces of a different cellular nature, were more variable but in no case was there evidence of an active and continued multiplication such as occurs in the nasal passages.

It is true that subcutaneous injection was regularly attended by a local reaction, but relatively few bacteria were demonstrable either microscopically or culturally about the site of injury. There was no apparent multiplication of the specific bacillus in the blood or in other locations following direct intravenous injection. Isolation of the bacillus from the heart's blood of a bird that had died following intraperitoneal injection indicates, however, that maintenance in the blood may occasionally occur. Neither subcutaneous nor intravenous injection was followed by a migration of the specific bacillus to the nasal passages.

Intraperitoneal injection was attended by an unexpected departure from the preceding findings. Although there was no indication that the specific bacillus multiplied to any extent in the peritoneal cavity, it was found that over half of the birds so injected were carrying that organism in the nasal passages. Heretofore the fowl coryza bacillus has never been encountered in the upper air passages of normal birds.

In most cases only a few organisms were present. There was no visible inflammatory response and no leucocytes were found on films. One bird, however, showed a definite coryza and the presence of numerous bacilli. The experimental birds were maintained under environmental conditions which precluded, in so far as was possible, the acquisition of the specific bacillus from the outside. Carriage of the bacilli from the peritoneal cavity to the nasal passages seems to be clearly indicated. The way in which this carriage was effected is not known. Blood cultures were regularly negative but this finding does not necessarily contraindicate a circulatory carriage. The possibility that they might be transported in cells which had engulfed them at the site of injection suggests itself but is quite without proof.

CONCLUSIONS

The mucous surfaces of the nasal passages and orbital sinuses appear to afford particularly favorable conditions for the development of the fowl coryza bacillus. Injected in the nasal tract, in any appreciable number, the bacilli regularly develop and may continue to exist for a considerable period of time in spite of an active inflammatory reaction on the part of the host.

The specific bacillus multiplies either sparsely or not at all when injected extranasally, regardless of the nature of the cellular surface with which it is brought in contact.

If the locus of injection is in communication with the upper air passages, as in the case of the trachea, internal ear, and orbital cavity, the bacilli may be carried there, even in the absence of a local development, and produce a coryza.

Introduction of the bacilli in loci not in communication with the upper air passages is followed by a nasal carriage only in the case of the peritoneal cavity. Following intraperitoneal injection, 7 of 12 birds showed the specific bacillus in the nasal passages and except in one instance without an accompanying inflammation.

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STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

IV. SUSCEPTIBILITY AFTER EXTRANASAL INJECTION OF THE FOWL CORYZA BACILLUS

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It was previously noted that recovery from coryza in the domestic fowl was often attended by an altered state of susceptibility.¹

Birds which had recovered from the coryza produced by the intranasal injection of exudate were generally resistant to reinfection with either exudate or the fowl coryza bacillus. Birds which had recovered from the coryza produced by injection of the specific bacillus were generally resistant to reinfection with the bacillus but were not completely protected against reinfection with exudate. The introduction of exudate was commonly followed by a coryza appearing after an incubation period of 10 days or longer. In these cases the specific bacillus was rarely isolated from the nasal passages. The injection of normal fowl with exudate from these birds was followed, in some instances, by an immediate return to the original coryza of rapid onset. In other instances, observed recently, the prolonged incubation period persisted with continued passage from bird to bird.

The experiments which are reported in the present paper were undertaken to determine whether or not the susceptibility of the fowl could be altered by extranasal injection of the specific bacillus.

EXPERIMENTAL

Intratracheal Injection.—Six birds which had shown no symptoms of coryza following intratracheal injection of the specific bacillus were injected intranasally after an interval of 14 days with the same organism.²

¹ Nelson, J. B., *J. Exp. Med.*, 1933, 58, 297.

² Unless otherwise specified, 0.5 cc. amounts of living, 24 to 48 hours old, chicken serum-agar cultures of the fowl coryza bacillus were used in these experiments.

The susceptibility of these 6 birds was identical with that of a previously untreated bird which was injected at the same time with the same inoculum. In each case, after an incubation period of 24 hours, a coryza of short duration was manifested by a nasal discharge from which the specific bacillus was readily isolated.

Intracloacal Injection.—Three birds were injected intracloacally with the fowl coryza bacillus and the same number with infective exudate. The birds were kept under observation for 17 and 14 days, respectively. They were then injected intranasally with the same inoculum.

The 6 birds were unaffected by the intracloacal injection. Their response to the intranasal injection was similar to that of 2 previously untreated birds which were injected simultaneously with culture fluid and exudate, respectively. The birds which had received an intranasal injection of the specific bacillus all developed a coryza of rapid onset and short duration. The incubation period was 24 hours in each case. The birds which had received an intranasal injection of exudate likewise showed a nasal discharge after 24 hours. In these birds the coryza was of long duration, inflammation of the nasal mucosa being still apparent 4 weeks later, when they were killed. The fowl coryza bacillus was regularly isolated from the nasal passages in both cases.

Subcutaneous Injection.—Susceptible fowl were injected subcutaneously in the outer surface of the fore leg with 1.0 cc. amounts of culture fluid. Five birds received a single injection and 3 received 3 injections which were spaced 4 days apart. After a rest period of 12 to 16 days they were injected intranasally with cultures of the specific bacillus.

The susceptibility of the 5 birds which had received a single subcutaneous injection of the fowl coryza bacillus was comparable with that of an untreated bird injected at the same time with the same culture fluid. After a short incubation period, varying from 24 to 48 hours, all of the birds showed a coryza of short duration. The specific bacillus was identified in cultures from the nasal exudate.

The 3 birds which had received 3 subcutaneous injections likewise showed an unaltered susceptibility. Intranasal injection of the fowl coryza bacillus was promptly followed by a nasal discharge.

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Intraperitoneal Injection.—Normal birds were injected intraperitoneally with cultures of the fowl coryza bacillus. Fifteen birds received a single injection and 10 were given 2 injections spaced 3 days apart. After a period of rest, during which time they remained normal, all of the birds in the first group and 5 in the second were given an intranasal injection of the specific bacillus. The 5 remaining birds in the second group were tested intranasally with exudate. Three other birds which had received the specific bacillus intranasally, in order to check the infectivity of the culture, were also tested, upon recovery, with exudate.

The results of these experiments are summarized in Tables I to III. Nine of the 15 birds which were given a single intraperitoneal injection of the specific bacillus showed no immediate symptoms of coryza following the intranasal injection. These birds were kept under observation for periods varying from 3 to 4 weeks and were then brought to autopsy. They showed no evidence of an involvement of the upper air passages during this time. Postmortem examination revealed no inflammatory manifestations in the nasal tract.

Four of the 5 birds which received 2 intraperitoneal injections likewise showed no response to the intranasal injection of the specific bacillus. These birds were held in quarantine for a period of 4 weeks and then brought to autopsy. Cultural examination of the nasal mucosa at this time failed to reveal the fowl coryza bacillus.

Six birds in the first group and one in the second showed a coryza of short duration which was first manifested, in each case, on the day following the intranasal injection. The specific bacillus was readily isolated from the nasal exudate. Two previously untreated birds which were given an intranasal injection of the bacillus likewise developed a coryza of rapid onset and short duration.

A single bird of the 5 which were injected intranasally with exudate following 2 intraperitoneal injections of the specific bacillus showed normal susceptibility. This bird, No. 25, developed a coryza on the 2nd day after injection. The specific bacillus was readily isolated from the nasal exudate. Three of the birds showed no immediate response to the injection but after a prolonged incubation period, varying from 14 to 27 days, they developed a nasal discharge from which the fowl coryza bacillus was not isolated. Exudate from one of these birds, No. 21, was subsequently passed through 2 additional birds. In each case a coryza of slow onset was produced and in each

case attempts to cultivate the specific bacillus from the nasal exudate met with failure. One bird, No. 24, showed no symptoms of coryza either immediately after injection or during a subsequent observation period of 4 weeks. At autopsy, the nasal mucosa was normal.

TABLE I
The Effect of Intraperitoneal Injection on Susceptibility to the Fowl Coryza Bacillus

Bird No.	Length of time to intranasal injection	Nasal reaction
	<i>days</i>	
1	11	—
2	11	—
3	14	+ 24 hrs.
4	14	—
5	14	—
6	11	+ 24 hrs.
7	11	+ 24 hrs.
8	11	+ 24 hrs.
9	11	+ 24 hrs.
10	11	+ 24 hrs.
11	11	—
12	11	—
13	11	—
14	11	—
15	11	—
16*	7	+ 24 hrs.
17	7	—
18	7	—
19	7	—
20	7	—

* The last 5 birds received 2 intraperitoneal injections.

A previously untreated bird which was injected intranasally with a portion of the same exudate used in testing for susceptibility showed a coryza on the following day. The fowl coryza bacillus was isolated from the nasal exudate.

One of the 3 birds which were injected with exudate following recovery from the coryza produced by the bacillus in pure culture showed normal susceptibility. In this bird, No. 26, the intranasal

injection was promptly followed by a nasal discharge from which the specific bacillus was isolated. Two of these birds, Nos. 27 and 28, showed an abnormal response characterized by a coryza of slow onset which was manifested by a nasal discharge on the 19th and 16th day, respectively. The specific bacillus was not isolated from the nasal exudate in either case.

TABLE II
The Effect of Intraperitoneal Injection on Susceptibility to Exudate

Bird No.	Length of time to intranasal injection	Nasal reaction
	<i>days</i>	
1	7	+ 14 days
2	7	+ 27 days
3	7	+ 27 days
4	7	- 30 days
5	7	+ 2 days

TABLE III
The Effect of Recovery from the Coryza Produced by Cultures of the Specific Bacillus on Susceptibility to Exudate

Bird No.	Incubation period	Duration of discharge	Length of time to reinfection	Nasal reaction
			<i>days</i>	
1	1 day	5 days	11	+ 2 days
2	1 day	6 days	11	+ 19 days
3	1 day	6 days	11	+ 16 days

DISCUSSION

Intratracheal and intracloacal injection of the fowl coryza bacillus had no apparent effect on the susceptibility of fowl to coryza. Growth of the specific bacillus was as well sustained in the nasal passages of birds which had previously been injected by these routes as it was in untreated birds of known susceptibility. The inflammatory response of the host was the same in both cases. These findings might be anticipated from the earlier noted observation that the fowl coryza bacillus develops only irregularly in the trachea and is unable to establish itself in the cloaca.³

³ Nelson, J. B., *J. Exp. Med.*, 1935, 61, 351.

Subcutaneous injection likewise failed to influence the susceptibility of fowl to coryza. Intranasal introduction of the specific bacillus promptly and regularly elicited a nasal discharge in birds which had received an earlier subcutaneous injection. It was previously noted that subcutaneous injection of the fowl coryza bacillus is regularly attended by a limited growth of the bacteria at the site and a local inflammatory reaction on the part of the host.³ Growth is apparently checked by this reaction before the bacilli have multiplied sufficiently to provoke a general immune response.

Intraperitoneal injection, however, was followed by an irregular but definite alteration in the susceptibility of the host to coryza. Thus, 9 of 15 birds (60 per cent) which had received a single intraperitoneal injection of the specific bacillus and 4 of 5 (80 per cent) which had received 2 injections were unaffected by a subsequent intranasal injection. Four of 5 birds that were subsequently injected with exudate likewise showed an altered susceptibility. The specific effect, in this case, was less conspicuous but was comparable to that attendant upon recovery from the coryza produced by cultures of the specific bacillus.

Introduction of the fowl coryza bacillus into the nasal passages of susceptible fowl is invariably followed by an active multiplication which is opposed by a vigorous local inflammatory reaction. Multiplication of the bacteria occurs largely, if not solely, outside the cells which line the nasal passages. As the result of intraperitoneal injection it appears that environmental conditions which normally favor development of the specific bacillus in the nasal tract are so altered that bacilli which are introduced there are either destroyed or held in check. This inhibition is accomplished without the intervention of an inflammatory response.

The earlier finding that intraperitoneal injection may be followed by a migration of the specific bacillus to the nasal tract is of interest in connection with this altered susceptibility of the fowl.³ The bacilli which have migrated to the upper air passages rarely multiply to any extent and are rarely opposed by a local inflammation. Thus, active multiplication of the bacillus, followed by a nasal discharge, was observed only once in a series of 37 birds injected intraperitoneally. The question arises whether the bacteria which are present in the

nasal passages as the result of migration from the peritoneal cavity may not be directly concerned with the altered reaction of the host. Stated in other words, is the altered state of susceptibility which is provoked by intraperitoneal injection referable to a local immunity induced by the few bacilli which are present in the nasal tract, or is it a local manifestation of a general immunity stimulated by the presence of the bacilli in the peritoneal cavity?

The fact that subcutaneous injection, which is followed by a limited development of the bacillus locally but is unattended by a migration to the nasal passages, has no demonstrable effect on the susceptibility of the fowl tends to favor a local as opposed to a general immunity. The rough approximation between the number of birds which are found to harbor the specific organism intranasally and the number which show an altered susceptibility, following intraperitoneal injection, also supports the view that a local process is involved. The present evidence, however, is too meager to warrant a conclusive answer to the question which has been raised.

SUMMARY

Intratracheal, intracloacal, and subcutaneous injection of living cultures of the fowl coryza bacillus had no demonstrable effect on the susceptibility of fowl to coryza.

Intraperitoneal injection was irregularly followed by a definitely altered susceptibility. Growth of the specific bacillus was inhibited in the nasal tract of approximately 70 per cent of 25 birds which had received an earlier intraperitoneal injection.

PHENOMENON OF LOCAL SKIN REACTIVITY TO BACILLUS TUBERCULOSIS

I. SKIN-PREPARATORY AND REACTING POTENCIES OF TUBERCULIN, O.T., AND BACILLUS TUBERCULOSIS CULTURE FILTRATES

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Since the phenomenon of local skin reactivity to bacterial filtrates was first described, attempts have been made to reproduce it with culture filtrates, different culture products and live cultures of a great variety of microorganisms including *B. tuberculosis* (1). Nasta reported failure to elicit the phenomenon with tuberculin, O.T. (2). According to Bieling, Oelrichs was able to obtain it with certain culture filtrates of *B. tuberculosis* (3). In her experiments, the phenomenon-producing factors were not related to the substances responsible for tuberculin hypersensitiveness. Bordet observed reactions in guinea pigs at the site of intradermal inoculations of BCG cultures following the intravenous injection of *B. coli* culture materials (4). Freund reported that intravenous injection of *B. typhosus* culture filtrate produced severe reactions at the site of intradermal tuberculin tests in guinea pigs sensitized with BCG cultures. He obtained no reactions in normal guinea pigs (5).

The object of the work embodied in this paper was to investigate further the phenomenon-producing properties of various preparations from cultures of tubercle bacilli, with special reference to processes of tuberculin hypersensitiveness.

Strains.—The strains of *B. tuberculosis* employed were the Bovine Type—C₁ 458-559 (of New York City Board of Health); Human Type—H₁₁; and Avian Type—823 of the American Type Culture Collection.

Cultures.—The strains employed were each seeded on the surface of 250 cc. of 5 per cent glycerine broth pH 7.2 placed in 1000 cc. Erlenmeyer flasks. The cotton plugs were sealed with paraffin and the cultures incubated at 37.5°C. The length of the incubation period is indicated in the tables.

Filtrates.—The cultures were centrifuged at high speed. The clear supernatant fluid was decanted and filtered through Berkefeld V candles. Sterility controls were made on plain agar slants and Lowenstein media.

Tuberculin, O.T.—Cultures were heated in the Arnold sterilizer for 1½ hours and filtered through one layer of sterile filter paper. The filtrates were transferred to sterile beakers and evaporated to 1:10 original volume in the water bath at 56°C. The length of time required for evaporation varied from 24 to 36 hours.

The experiments described in this part of the paper deal with attempts to elicit the phenomenon of local skin reactivity to *B. tuberculosis* in normal rabbits by the utilization of products of the bacilli for intradermal and intravenous injections.

In preliminary experiments, the primary irritating effect of intradermal injections of tuberculin, O.T., and various culture filtrates was studied. Filtrates gave no local reactions. Undiluted tuberculin frequently produced pustules with hemorrhagic zones at the periphery 24 hours after intradermal injections. Dilutions 1:3 elicited small pustules with a bright red erythema at the periphery. Dilutions 1:5 and higher gave no noticeable inflammatory response. The irritation described was apparently due to the high concentration of glycerine, since preparations of 25 and 50 per cent glycerine in plain sterile broth gave similar reactions. In the experiments which follow, it seemed advisable to avoid primary skin irritation, since it was previously observed that in instances in which the preparatory factors produced no primary reaction the lesions following intravenous injections of bacterial filtrates were clearly defined and stronger than when the intradermal injections by themselves elicited inflammation.

As is seen in Table I, an undiluted Filtrate 1703 and several batches of tuberculin, O.T., diluted 1:7.5 were employed for preparation of the skin. 24 hours later, large doses of the filtrates and tuberculin were injected intravenously. No reactions were to be seen 4 and 24 hours after the intravenous injections.

In this part of the work advantage was taken of the fact that the skin prepared with the factors of one microorganism reacts to the intravenous injection of cultures and culture filtrates of other unrelated microorganisms (6).

As is seen in Table II, the skin of rabbits was prepared by single injections of 0.25 cc. of various batches of human and bovine tuberculin, O.T., a filtrate of bovine *B. tuberculosis* broth culture and purified tuberculin T.P.T. (kindly supplied by Dr. Florence Seibert). 24 hours later, the rabbits received single intravenous injections of

meningococcus and *B. typhosus* "agar washings" filtrates. The potency of the filtrates, as indicated in Table II, was determined on rabbits prepared by intradermal injections of homologous filtrates.

In these experiments, batches of human and bovine tuberculin, O.T., prepared in the Mount Sinai Hospital Laboratories were capable of inducing the state of reactivity (Groups 1, 4, 5, 6 and 8). Similar attempts with the New York Board of Health tuberculins and with

TABLE I

Phenomenon of Local Skin Reactivity to B. tuberculosis by the Use of Tuberculous Substances for Intradermal and Intravenous Injections in Normal Rabbits

Group No.	Skin-preparatory injections*		Intravenous injections		Reactions 4 and 24 hrs. after intravenous injections
	Material used	Dilution	Material used	Dose per kg. of weight	
1	Human Tuberculin, O.T., 12 Mt. Sinai	1:7.5	Human Tuberculin, O.T., 12 Mt. Sinai	1 cc. undiluted	0/3†
2	" "	1:7.5	" "	1 cc. diluted 1:2	0/3
3	1703-Human Tb. H ₂ —filtrate of 5% glycerine broth culture 6 wks. old	Undiluted	1703-Human Tb. H ₂ —filtrate of 5% glycerine broth culture	3 cc. undiluted	0/9
4	Human Tuberculin, O.T., 14 N. Y. Board of Health	1:6	Human Tuberculin, O.T., 14 N. Y. Board of Health	2 cc. diluted 1:2	0/4

* In all the tables, 0.25 cc. was injected intradermally.

† In all the tables, the numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum of both indicates the total number of rabbits in each group.

the purified T.P.T. tuberculin consistently failed. It becomes obvious then that the skin-preparatory factors are found in tuberculins in low and variable concentrations.

The previously described (7) reciprocal quantitative relationship between the skin-preparatory and reacting factors is observed in these experiments as well. Thus, Tuberculin 12 diluted 1:7.5 reacted with 10 reacting units. When diluted, it failed to react with less than 15 units. Also, 25 reacting units did not elicit reactions at sites prepared with Tuberculin 10 dilutions higher than 1:35.

In experiments summarized in Table III, meningococcus and *B. typhosus* culture filtrates of ascertained skin-preparatory potency

TABLE II

Phenomenon of Local Skin Reactivity to B. tuberculosis by the Use of Tuberculous Products for Intradermal Injections, and Heterologous Bacterial Filtrates for Intravenous Injections

Group No.	Skin preparatory injection		Intravenous injection		Reaction 5 hrs. after intravenous injection
	Material used	Dilution	Material used	Dose per kg. of weight	
1	Bovine Tuberculin, O.T., 10 Mt. Sinai	Diluted 1:5	T.1837 B.TyT _L *	25 units	3/0
2	" "	" 1:7.5	" "	25 "	0/5
3	" "	" 1:7.5	T.1832 B.TyT _L	25 "	0/3
4	" "	" 1:5	T.1836 Mg.44B.†	25 "	2/1
5	" "	" 1:15	T.1847 B.TyT _L	25 "	0/3
6	Human Tuberculin, O.T., 12 Mt. Sinai	" 1:7.5	T.1837 B.TyT _L	10 "	3/0
7	" "	" 1:15	" "	12.5 units	0/3
8	" "	" 1:5	" "	15 units	1/2
9	T.P.T. purified tuberculin Seibert	10 mg.	" "	15 "	0/3
10	N. Y. Board of Health Tuberculin, O.T., 67	Diluted 1:3	" "	15 "	0/3
11	" "	Diluted	" "	15 "	0/3
12	Filtrate of 10 day broth culture of human Tb. 1800	Undiluted	T.1795 Mg. 44B.	15 "	0/3
13	Bovine Tuberculin, O.T., 7 Mt. Sinai	Diluted 1:7.5	T.1815 B.TyT _L	25 "	0/3
14	N. Y. Board of Health Tuberculin, O.T., 14	" 1:5	T.1876 Mg.44B.	25 "	0/3
15	" "	" 1:5	" "	40 "	0/3

The titrations of the reacting factors were made with homologous filtrates injected intradermally.

* In this and the following tables, abbreviation B.TyT_L designates "agar washings" filtrates of *B. typhosus*, Strain T cultures.

† In this and the following tables, abbreviation Mg.44B. designates "agar washings" filtrates of meningococcus Group III cultures.

were employed for intradermal injections. Various batches of tuberculin, O.T., were injected intravenously into the rabbits 24 hours later.

Here again, severe reactions resulted from five tuberculin batches (bovine Tuberculin, O.T., 2, 3, 5, 11, and human Tuberculin 12)

TABLE III

Phenomenon of Local Skin Reactivity to B. tuberculosis by the Use of Heterologous Bacterial Filtrates for Intradermal Injections and Tuberculous Substances for Intravenous Injections

Dose and material used for skin-preparatory injections	Preparation employed for intravenous injections	Dose of intravenous injection per kg. of weight	Results
0.25 cc. T.1795 Mg.44B.	50% glycerine broth	4 cc. undiluted	0/3 (one died 1 min. after intravenous injection)
0.25 " " "	" "	3 " "	0/3
0.25 " " "	" "	2 " "	0/3
0.25 " T.1787 "	Human Tuberculin, O.T., 2 Mt. Sinai	1.2 " "	1/0
0.25 " " "	Bovine Tuberculin, O.T., 3 Mt. Sinai	1 cc. dilution 1:2	3/0
0.25 " " "	" "	1 " " 1:7	0/3
0.25 " T.1803 B.TyT ₂	Bovine Tuberculin, O.T., 5 Mt. Sinai	1 " undiluted	2/1
0.25 " " "	" "	1 " dilution 1:2	1/1
0.25 " T.1815 "	Human Tuberculin, O.T., 6 Mt. Sinai	1 " undiluted	0/3
0.25 " " "	Avian Tuberculin, O.T., 9 Mt. Sinai	1 " "	0/3
0.25 " T.1832 "	Bovine Tuberculin, O.T., 10 Mt. Sinai	1 " "	0/3
0.25 " " "	Bovine Tuberculin, O.T., 11 Mt. Sinai	1 " "	3/0
0.25 " " "	" "	1 " dilution 1:2	1/2
0.25 " " "	Human Tuberculin, O.T., 12 Mt. Sinai	1 " undiluted	2/1
0.25 " " "	" "	1 " dilution 1:2	3/0
0.25 " " "	" "	1 " " 1:5	0/3

but failed with three other preparations (human Tuberculin 6, avian Tuberculin 9 and Bovine tuberculin 10). It is of interest that bovine

Tuberculin 10, which failed to elicit reactions in the experiments revealed here, proved potent when used as a skin-preparatory agent.

As is seen from the experiments thus far described, tuberculin, O.T., contains in low concentration the factors necessary for the elicitation of the phenomenon. This becomes obvious if the doses employed in these experiments are compared with those of meningococcus, *B. typhosus* and other culture filtrates. The reactions under discussion cannot be obtained unless non-tuberculous bacterial filtrates of high potency are used either for the skin preparation or for intravenous injection. In this manner the deficiency in potency of tuberculin is made up for by taking advantage of the strict quantitative reciprocity of skin-preparatory and reacting (intravenous) factors recorded in previous papers.

It is noteworthy that a batch of tuberculin was encountered which was endowed with skin-preparatory potency but was lacking in reacting factors. This is also a corroboration of previous observations on the apparent independence of skin-preparatory and reacting factors. Thus, the majority of *B. typhosus* culture filtrates contained about 150 skin-preparatory units and between 400 to 600 reacting units per 1 cc. On the other hand, some meningococcus culture filtrates contained as many as 3000 reacting units for the same or smaller numbers of skin-preparatory units. Moreover, culture filtrates of some bacterial species (*i.e.* streptococcus) (6), serum precipitates (8), agar (9, 8) and starch (5) empowered with reacting potency were devoid of skin-preparatory effect.

In the experiments reported here, Seibert T.P.T. and the New York Board of Health tuberculin were completely inactive whilst preparations made in these laboratories were capable of eliciting the phenomenon. This demonstrates that the factors necessary for the elicitation of the phenomenon of local skin reactivity are not related to tuberculin substances proper.

As described above the tuberculin of these laboratories was prepared by evaporation at 56°C. for 24 to 36 hours. The New York Board of Health workers evaporated the tuberculin by boiling over a free flame. It was shown in our early publications that prolonged exposure to heat is likely to inactivate the factors necessary for the phenomenon of local skin reactivity under discussion. Presumably, therefore, the lack of reacting potency in the New York Board of Health preparation

may be due to prolonged boiling. Apparently, the purification methods of Seibert also remove the toxic substances described here.¹

Recent studies on the phenomenon of local skin reactivity to bacterial filtrates have demonstrated that the intravascular interaction of animal proteins and bacterial antigens with homologous antibodies brings about prompt and severe hemorrhagic necrosis at the site of tissues rendered vulnerable by previous injection of a potent bacterial filtrate. The interaction of antigens with antibodies was obtained as follows: (1) By the use of mixtures made *in vitro* of animal sera and egg albumin with homologous antibodies; (2) through intravenous injection of animal sera and egg albumin into rabbits passively sensitized with homologous antisera; (3) by the intravenous injection of animal proteins into rabbits previously actively sensitized to the same protein, and (4) through the intravenous injection of pneumococcus culture filtrates, incapable of eliciting the phenomenon by themselves, into rabbits previously passively sensitized with antipneumococcus antisera (10), and, as also recently shown by Plaut (11), through the intravenous injections of mixtures of *Spirocheta pallida* haptenes with anti-*Spirocheta* serum.

The object of the experiments reported in this paper was to determine whether the interaction of tuberculous material with homologous antibodies would elicit hemorrhagic necrosis at sites prepared by injection of potent bacterial filtrates.

(a) Fifteen groups of three rabbits were given each, weekly single intradermal injections of bacterial filtrates of ascertained skin-preparatory potency. 24 hours after each intradermal injection the rabbits

¹In recent publications (5) Freund has stated that the primary hemorrhagic effect of substances injected intradermally is in direct relationship to its phenomenon-producing potency. This conclusion would appear to be erroneous. For as can be seen from the experiments described in the present paper, tuberculin, O.T., has a primary hemorrhagic effect associated with a low skin-preparatory potency. Moreover, the skin-preparatory potency may be absent from many batches of tuberculin, whilst the primary hemorrhage-producing property, which is due to high concentration of glycerine, is constantly present.

In extensive histological studies on the phenomenon, Gerber (13) found that *B. typhosus* culture filtrates with a skin-preparatory potency shown above to be ten to twenty times stronger than tuberculin never elicited any primary hemorrhages. Additional experiments dealing with this problem will be published in the near future.

Group No.	Skin-preparatory injections						1st
	1st	2nd	3rd	4th	5th	6th	
1	0.25 cc. T.1811 Mg.44B.	0.25 cc. T.1815 B.TyT _L	0.25 cc. T.1826 Mg. 20745*	0.25 cc. T.1815 B.TyT _L	0.25 cc. T.1815 B.TyT _L	0.25 cc. T.1832 B.TyT _L	3 cc. T.1819 bo- vine Tb. fil- trate 4 wks. old
2	" "	" "	" "	" "	" "	" "	" "
3	" "	" "	" "	" "	" "	" "	3 cc. T.1818 bo- vine Tb. fil- trate 8 wks. old
4	" "	" "	" "	" "	" "	" "	3 cc. T.1817 bo- vine Tb. fil- trate 9 wks. old
5	" "	" "	" "	" "	" "	" "	3 cc. T.1827 bo- vine Tb. fil- trate 5 days old
6	" "	" "	" "	" "	" "	" "	1 cc. Tuberculin 7 bovine, O.T.
7	" "	" "	" "	—	—	—	1 cc. Tuberculin 5 bovine, O.T.
8	" "	" "	" "	0.25 cc. T.1815 B.TyT _L	0.25 cc. T.1815 B.TyT _L	0.25 cc. T.1815 B.TyT _L	3 cc. T.1825 hu- man Tb. fil- trate 4 wks. old
9	" "	" "	" "	" "	" "	" "	3 cc. T.1824 hu- man Tb. fil- trate 5 wks. old
10	" "	" "	" "	" "	" "	" "	3 cc. T.1823 hu- man Tb. fil- trate 9 wks. old
11	" "	" "	" "	" "	" "	" "	1 cc. human Tuberculin 6
12	" "	" "	" "	" "	" "	" "	3 cc. T.1828 avian Tb. fil- trate 5 days old
13	" "	" "	" "	" "	" "	" "	3 cc. T.1822 avian Tb. fil- trate 4 wks. old
14	" "	" "	" "	" "	—	—	3 cc. T. 1820 av- ian Tb. filtrate
15	" "	" "	" "	" "	0.25 cc. T.1815 B.TyT _L	0.25 cc. T.1815 B.TyT _L	1 cc. Tuber- culin 9 av- ian, O.T.

* Mg. = meningococcus Strain 20745.

Intravenous injections

3rd	4th	5th	6th	7th	Reactions 5 hrs. after intravenous injections						
					1st	2nd	3rd	4th	5th	6th	7th
cc. T.1819	3 cc. T.1819	3 cc. T.1819	3 cc. T.1819	—	0/3	0/3	0/3	0/3	0/2	0/1	—
" "	3 " "	3 " "	1 cc. human Tuberculin, O.T., 6	—	0/2	0/2	0/2	0/2	0/2	2/0	—
" T.1818	3 " T.1818	3 " T.1818	3 cc. T.1818	—	0/3	0/3	0/3	0/3	0/3	0/3	—
" T.1817	3 " T.1817	3 " T.1817	3 " T.1817	—	0/3	0/3	1/2	1/2	1/2	3/0	—
" T.1827	3 " T.1827	3 " T.1827	3 " T.1827	—	0/3	0/3	0/3	0/3	0/3	0/3	—
cc. Tuberculin 7 bovine, O.T.	1 cc. Tuberculin 7 bovine, O.T.	1 cc. Tuberculin 7 bovine, O.T.	1 cc. Tuberculin 7 bovine, O.T.	—	0/3	0/3	0/2	0/2	0/2	0/2	1/1
cc. T.1825	3 cc. T.1825	3 cc. T.1825	3 cc. T.1825	—	0/3	0/3	3/0	—	—	—	—
" T.1824	3 " T.1824	3 " T.1824	3 " T.1824	1 cc. human Tuberculin 6	0/3	0/3	0/2	0/2	0/2	0/2	1/1
" T.1823	3 " T.1823	3 " T.1823	3 " T.1823	" "	0/3	0/3	0/2	0/2	0/2	0/2	1/1
cc. Tuberculin 6	1 cc. Tuberculin 6 dilution 1:10	1 cc. Tuberculin 6 dilution 1:10	—	—	0/3	0/3	0/2	0/2	0/2	0/2	1/1
cc. T.1828	3 cc. T.1828	3 cc. T.1828	3 cc. T.1828	—	0/3	1/1	2/0	0/2	0/2	—	—
" T.1822	3 " T.1822	3 " T.1822	3 " T.1822	1 cc. human Tuberculin 6	0/3	0/3	0/3	0/3	0/3	0/3	0/2
" T.1820	3 " T.1820	3 " T.1820	—	3 cc. T.1817	0/3	0/3	0/3	0/3	0/3	1/2	3/0
cc. Tuberculin 9	1 cc. Tuberculin 9	1 cc. Tuberculin 9 dilution 1:5	1 cc. Tuberculin 9 dilution 1:10	—	0/3	0/3	0/3	2/1	0/2	—	—
				—	0/3	0/3	0/3	2/1	1/2	0/1	0/2

were injected intravenously with certain tuberculin, O.T., batches and filtrates of tuberculous cultures of various ages. The results are summarized in Table IV.

Following the first intravenous injection of the materials selected, no reactions appeared at the prepared sites. After the second injection, there appeared reactions in one group (No. 11). The third injection produced necrosis again in Group 11 and also in Groups 4 and 7. As a result of the fourth injection, reactions were obtained in previously susceptible Group 4, and in new Groups 14 and 15. The fifth injection produced repeated reactions in Groups 4 and 15; the sixth injection repeated reactions in Groups 4 and 15 and in previously non-reactive Groups 2 and 13, and finally the seventh injection elicited necrosis in new Groups 6, 8, 9 and 10 and the previously reactive Group 4. As is also seen in the same table, the reactions elicited in the manner described were obtained with tuberculin, O.T., preparations of human, bovine and avian types, and bovine, human and avian *B. tuberculosis* culture filtrates of 4 and 9 weeks incubation, and failed with two 5 day and one 8 week old culture filtrates of bovine type.

It is also of interest that some of the groups once reactive continued to show reactions with each subsequent intravenous injection (Groups 4, 13), whilst others became refractory after one or several reactions (Groups 11, 14, 15).

As is seen from all the facts presented, it is possible to elicit the phenomenon of local skin reactivity to bacterial filtrates by repeated weekly intravenous injections of tuberculous materials into rabbits prepared by intradermal injections of bacterial filtrates of ascertained skin-preparatory potency (meningococcus, *B. typhosus*, etc.). The materials may be either tuberculin, O.T., preparations of human, bovine and human types or culture filtrates of 4 and 9 weeks incubation. The failure to elicit reactions with 5 day and 8 week old culture filtrates may be due to a natural variation in susceptibility of rabbits.

After varying intervals of time, *i.e.* from 1 to 3 weeks, some rabbits may become refractory to the phenomenon. It remains to determine whether the preparations vary in their ability to induce the refractory state.

(b) Attempts were made to elicit reactions by injection of mixtures made *in vitro* of tuberculous materials with homologous antisera and

also by the intravenous injection of tuberculous material into rabbits passively sensitized by previous injection of tuberculous antisera. All the rabbits were prepared with meningococcus and *B. typhosus* "agar washings" filtrates 24 hours prior to the intravenous injection. The experiments with *in vitro* mixtures are summarized in Table V.

There were also numerous experiments on passively sensitized rabbits not recorded here. The antisera were sera of rabbits showing reactions in experiments of Table IV; sera of human cases of tuber-

TABLE V

Phenomenon of Local Skin Reactivity to B. tuberculosis by the Use of Heterologous Bacterial Filtrates for Intradermal Injections and Mixtures of Tuberculous Substances with Antibodies for Intravenous Injections in Normal Rabbits

Group No.	Skin-preparatory injection	Intravenous injection			Results
		Ingredients of mixtures		Dose of mixtures	
1	0.25 cc. T.1838	Tuberculin 12	R. Serum 559*	2	0/3
2	Mg.44B.	dilution 1:6			
3	" "	Tuberculin 12	" " 559	2	0/3
4	" "	dilution 1:4			
5	" "	Tuberculin 12	" " 577	2	3/0
6	" "	dilution 1:6			
		" "	" " 573	2	0/3
		" "	" " 577	2	0/3
			dilution 1:50		
		Tuberculin 12	R. Serum 577	2	1+/2
		dilution 1:10			

* R. = rabbit.

culosis and tuberculous guinea pigs. With the exception of one instance recorded in Table IV, all the experiments failed. For the time being, therefore, it appears that in contradistinction to experiments with pneumococcus, passively acquired tuberculous antigen-antibody complexes are incapable of eliciting reactions at the prepared skin sites.

DISCUSSION AND CONCLUSIONS

In this paper there is described the phenomenon of local skin reactivity to *B. tuberculosis*. The skin-preparatory and reacting factors

of this phenomenon may be obtained in tuberculin, O.T. However, because of their low concentration, it is necessary to inject either intradermally or intravenously heterologous bacterial filtrates of high potency. Thus, advantage is taken of the previously observed quantitative reciprocity of the skin-preparatory and reacting factors.

The skin-preparatory and reacting factors of tuberculin have no apparent relation to the tuberculin substances proper, inasmuch as standardized tuberculins of New York Board of Health and Seibert T.P.T. tuberculin fail to elicit the phenomenon of local skin reactivity to *B. tuberculosis*. The preparations which were potent were made in these laboratories by evaporation at 56°C. for 24 to 48 hours. It is possible that the skin-preparatory and reacting factors are destroyed by the higher temperatures employed for preparation by the Board of Health; and also by the Seibert chemical methods for purification.

The demonstration of certain new toxic substances in tuberculous materials seems to be of interest. Experiments are under way in order to determine whether the toxic substances can be specifically neutralized in a manner similar to meningococcus and *B. typhosus* toxic substances.

Preparations which fail to elicit reactions on a single intravenous injection may be able to do so on weekly repeated intravenous injections. (A detailed description is given on pages 375 and 376.) There is no direct proof as to the underlying mechanism. However, it appears possible that it depends on the interaction of the tuberculin with antibodies actively acquired after repeated injections. This was clearly shown to be the case with active animal proteins and other bacterial antigens by passive transfer experiments.

It appears, therefore, that the observations can be advantageously used as a measure of antibody response to tuberculous materials. The method is worthy of consideration because of the well known difficulties in demonstration of tuberculous antibodies. The observations present also an interest from the point of view of problems of tuberculin hypersensitiveness. As shown by Baldwin, Zinsser, *et al.*, (12) the essential prerequisite for elicitation of tuberculin hypersensitiveness is the presence of a tuberculous focus in the animal tissue, produced by live tubercle bacilli and more irregularly by massive doses of dead ones. Attempts to elicit this state with tuberculous culture

filtrates and tuberculin consistently failed. The experiments described here demonstrate a state of tuberculin hypersensitiveness of rabbits obtained with tuberculin and bacteria-free culture filtrates in the absence of tuberculous foci. The reactions can be elicited provided two important requirements are fulfilled: (1) The tissues are rendered vulnerable through contact with certain soluble bacterial filtrates. (2) The tuberculin or the tuberculous culture filtrates are injected intravenously into immunized rabbits.

It remains to determine whether the rôle of an active infection necessary for the tuberculin hypersensitiveness consists in the secretion of potent soluble preparatory factors having the properties described.

As has been seen, injury may be obtained in tissues rendered vulnerable by tuberculous materials, through the intravascular introduction of heterologous bacterial factors. This seems to point to a possible influence of secondary infections upon the evolution of tuberculosis and possibly may explain sudden relapses and exacerbations of chronic tuberculosis. It is planned to determine whether prophylactic immunization with the toxic substances derived from secondary organisms may prove beneficial in the care of tuberculosis.

SUMMARY

New toxic substances in certain tuberculin, O.T., and *B. typhosus* culture filtrates are described. These substances are capable of eliciting the hemorrhagic necrosis characteristic of the phenomenon of local skin reactivity provided heterologous bacterial filtrates of high potency are used either for the intradermal or the intravenous injection. The toxic substances apparently have no relationship to the tuberculin substances proper.

The experiments with inactive preparations also demonstrate in rabbits a state of hypersensitiveness to tuberculin, O.T., and bacteria-free culture filtrates in the absence of tuberculous foci. The reactions are elicited provided the tissues are rendered vulnerable through contact with certain soluble bacterial factors capable of eliciting the phenomenon of local skin reactivity to bacterial filtrates, and provided the tuberculin or the tuberculous culture filtrates are injected intravenously into immunized rabbits.

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GRADING OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES

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In attempting to define and elucidate the nature of local skin reactivity to bacterial filtrates it is of prime importance that methods be established for quantitative measurements of the reactivity. Experiments designed for this purpose and their possible application to studies on the relationship of the phenomenon under discussion to certain reactions of skin hypersensitiveness, are embodied in this paper.

Duration of Local Skin Reactivity to Bacterial Filtrates in Normal Rabbits

In early experiments on the phenomenon of local skin reactivity to bacterial filtrates it was reported that the state of reactivity disappears after 32 hours (1). Filtrates of 6 day old cultures of *B. typhosus* in tryptic digest broth were employed. Later in the work, "agar washings" filtrates were introduced, inasmuch as they were shown to contain toxic substances of considerably higher potency (2). The purpose of the work described in this part of the present paper was to study the duration of the state of reactivity induced by various preparations.

The skin of normal rabbits was prepared by a single intradermal injection of 0.25 cc. of *B. typhosus* or meningococcus culture filtrate, and after various intervals of time bacterial filtrates of ascertained reacting potency were injected intravenously. The results are summarized in Table I.

As is seen from Table I, the state of skin reactivity induced by *B. typhosus* tryptic digest broth culture filtrates disappears in 48 hours. Skin sites prepared with *B. typhosus* "agar washings" filtrates retain the reactivity for 72 hours but lose it after 96 and 120 hours. Men-

ingococcus 'agar washings' filtrates induce the state for as long a period as 96 hours and occasionally for 120 hours. Skin sites prepared with *B. typhosus* "agar washings" filtrates, previously heated at

TABLE I
Duration of Local Skin Reactivity to Bacterial Filtrates

Skin-preparatory injections *	Material used for intravenous injection	Dose of intravenous injection	Interval of time between skin-preparatory and intravenous injection	Results	Reacting units per cc. of filtrate
T.1832 B.Ty T _L †	T.1832 B.Ty T _L	25 reacting units	hrs.		
" "	" "	25 " "	24	3/0†	550
T.1832 B.Ty T _L heated 60°—1 hr.	" "	25 " "	72	1/2	550
T.1832 B.Ty T _L heated 100°—20 min.	0.25 cc. T.1832 B.Ty T _L heated 100°—20 min.	25 " "	48	3/8	—
" "	" "	25 " "	24	2/3	250
T.1815 B.Ty T _L	T.1815 B.Ty T _L	25 " "	48	0/3	250
" "	" "	25 " "	48	3/0	625
T.1834 B.Ty T _L tryptic digest broth culture filtrate (6 days of incubation)	T.1834 B.Ty T _L tryptic digest broth culture filtrate (6 days incubation)	25 " "	72	1/2	625
" "	" "	1 cc. undiluted	24	3/0	200
" "	" "	1 " "	48	0/3	200
" "	T.1832 B.Ty T _L	25 reacting units	24	3/0	—
" "	" "	25 " "	48	1/2	—
" "	" "	25 " "	72	0/3	—
T.1826 Mg.20745§	T.1826 Mg.20745	25 " "	24	3/0	950
" "	" "	25 " "	48	2/1	950
" "	" "	25 " "	72	2/1	950
" "	" "	25 " "	96	4/6	950
" "	" "	25 " "	120	1/2	950

* 0.25 cc. was injected intradermally.

† Abbreviation B.Ty T_L designates "agar washings" filtrates of *B. typhosus*, Strain T_L cultures.

‡ The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum of both indicates the total number of rabbits used in each group.

§ Abbreviation T.1826 Mg. designates "agar washings" filtrates of meningococcus cultures. The strain employed (No. 20745) was isolated from a case of cerebral spinal meningitis in this hospital.

100°C. for 20 minutes do not react for periods longer than 24 hours. Thus, the duration of local skin reactivity depends upon the preparation employed, *i.e.*, microorganism, method of preparation, etc.

As is also seen from Table I, there was determined the number of reacting units in the various preparations employed (3). Comparison of the titers with the above described results demonstrates clearly a strict parallelism between the reacting potency and the duration of reactivity induced. Preparations of low reacting potency (*B. typhosus*, tryptic digest broth culture filtrates and heated "agar washings" filtrates, containing 200 and 250 units, respectively) induce a state of reactivity of 24 hours duration. In contrast to this, *B. typhosus* "agar washings" filtrates containing 550 and 625 units elicit the reactivity for a period of 72 hours. When the intradermal injection of a *B. typhosus* preparation containing 200 units (T. 1834) is combined with the intravenous injection of "agar washings" filtrate, the reactivity lasts for 48 hours. Meningococcus "agar washings" filtrates containing 950 reacting units produce the most protracted state of reactivity (*i.e.*, 96 hours, occasionally 120 hours).

Duration of Local Skin Reactivity to Antigen-Antibody Complexes

It was previously reported (4) that the skin sites prepared by bacterial filtrates also undergo severe hemorrhagic necrosis when acted upon by toxic principles resulting from intravascular interaction of non-toxic antigens (*i.e.*, horse serum, egg albumin, etc.) with homologous antibodies. The interaction can be obtained in one of the following ways: by separate intravenous injection of antigen and the antibody; by intravenous injection of antigen into rabbits possessing actively acquired homologous antibodies; by injection of antigen into a site prepared by a bacterial filtrate with simultaneous intravenous injection of the antibody; and by injection of the antigen into the prepared skin area in rabbits possessing actively acquired antibodies. In the latter case, there apparently occurs intravascular formation of the toxic principles at the site of the locally injected antigen with the circulating actively acquired antibodies.

It was of interest to study the duration of local skin reactivity induced by "agar washings" bacterial filtrates to toxic principles formed *in vivo* through antigen-antibody interaction. Experiments were carried out as follows:

Rabbits were sensitized by single or repeated intravenous injections of horse serum. After various intervals of time indicated in Table II there were made single skin-preparatory injections of bacterial filtrates. The test injections of horse serum were given either locally into the prepared skin site or intravenously. The intervals of time between the skin-preparatory and test injections varied from 24 to 144 hours. When repeated skin-preparatory injections in the same rabbits were necessary, various bacterial filtrates were employed in order to avoid the acquirement of active immunity to the phenomenon under consideration (5). The results of the experiments are summarized in Table II.

As is seen from Table II, the reactivity induced by bacterial filtrates in horse serum-sensitized rabbits was not longer than in normal rabbits. The state elicited by meningococcus "agar washings" filtrates lasted for longer periods of time than with similar preparations of *B. typhosus*. It even appeared that the duration was decreased somewhat by previous sensitization with horse serum, since some groups prepared with meningococcus "agar washings" filtrates did not react 96 hours later. Inasmuch as the purpose of the work was to establish whether the duration of reactivity was increased by horse serum sensitization, more detailed investigation of the suggestive shortening was not made and, therefore, the question is left open.

It was also clearly seen that repeated sensitization with horse serum (1 to 3 weekly intravenous injections) did not prolong the duration of reactivity elicited by bacterial filtrates.

It is of interest to report the following experiment not recorded in Table II.

Three rabbits received each one intravenous injection of 1 cc. per kilo of body weight of normal horse serum. 48 hours later, 0.25 cc. of *B. typhosus* "agar washings" filtrates was given intradermally. 120 hours later the skin site injected with the *B. typhosus* filtrate and one unprepared skin site were each injected with 0.5 cc. of undiluted horse serum. The latter procedure was repeated four times. Following the first, second and third intradermal injections of normal horse serum into the sites prepared with bacterial filtrates marked hemorrhagic and necrotic lesions were obtained. No reactions were obtained from the injection of horse serum into unprepared sites. Following the fourth injection of horse serum, hemorrhagic necrosis appeared in all the horse serum-injected sites, which obviously were reactions of the Arthus phenomenon.

It is plain that the reactions obtained in the sites prepared by bacterial filtrates are elicited in rabbits before they become sensitive to

TABLE II

Duration of Local Skin Reactivity to Reacting Factors Formed *in Vivo*

Intradermal injections			Skin preparatory injections*			Interval of time between 1st sensitizing and various skin-preparatory injections			Test injections 24 hrs. after intradermal injections of bacterial filtrates			Interval of time between skin-preparatory and following test injections			Results of test injection		
1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
1 cc. horse serum	—	—	T.1826 Mg.†	T.1826 Mg.	T.1832 B.Ty T _L †	7	14	18	i. d. 0.5 cc. horse serum " "	i. d. 0.5 cc. horse serum " "	i. d. 0.5 cc. horse serum " "	48 72	—	—	—	—	—
" "	—	—	T.1832 B.Ty T _L " "	T.1836 Mg. T.1826 Mg.	— —	10	20	—	i. v. 1 cc. horse serum " "	i. v. 1 cc. horse serum " "	i. v. 1 cc. horse serum " "	72	96	120	2/1	2/0/0/2	—
" "	" "	" "	" "	" "	" "	7	14	—	" "	" "	" "	48	72	—	3/0	0/3	—
" "	" "	" "	" "	" "	" "	7	14	—	" "	" "	" "	96	120	—	0/3	0/3	—
" "	1 cc. horse serum	horse serum	" "	" "	T.1836 Mg.	7	14	21	" "	" "	" "	120	120	0/3	0/3	0/3/0/3	—

i. d. = intradermally, i. v. = intravenously.

* 0.25 cc. was injected intradermally.

† Abbreviation Mg. designates "agar washings" filtrates of meningococcus cultures.

‡ Abbreviation B.Ty T_L designates "agar washings" filtrates of *B. typhosus*, Strain T_L cultures.

§ The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The

sum of both indicates the total number of rabbits used in each group.

the Arthus phenomenon. The preparation with the bacterial filtrates by no means accelerates the process of sensitization to this phenomenon. Once the Arthus sensitization is obtained, the previous preparation by bacterial filtrates seemingly does not enhance the severity of the reactions.

Quantitative Measurements of Local Skin Reactivity of Normal and Horse Serum-Sensitized Rabbits

In previous experiments (2) the titration of the skin-preparatory potency of the filtrates was made as follows:

From four to six skin sites of rabbits were injected simultaneously with various dilutions of the filtrate tested. 24 hours later the rabbits received a single intradermal injection of a given amount of the filtrate. The experiment was repeated several times and the average minimal amount of the filtrate necessary for skin preparation was computed. In view of the previously recorded quantitative reciprocal relation existing between the skin-preparatory and reacting doses, it was necessary to inject large amounts of the filtrate intravenously in order to obtain reactions in several prepared skin sites. The injection of large amounts was troublesome because of the high mortality induced.

In the experiments described below, only one skin site was prepared. Each group of the rabbits tested received a different dilution of the filtrate. The intravenous dose was kept constant.

As is seen from Table III, the titrations of skin-preparatory factors of *B. typhosus* "agar washings" filtrates were tested in normal and horse serum-sensitized rabbits. The sensitization was accomplished by the intravenous injection of horse serum in a dose of 1 cc. per kilo of body weight. The injections were repeated until severe reactions appeared at skin sites prepared by bacterial filtrates 4 to 5 hours after the intravenous injection of the horse serum. Most of the rabbits acquired this sensitization 1 week after the first intravenous injection of horse serum. In some rabbits, two horse serum injections were required.

As is seen from the experiments summarized in Table III, dilutions of "agar washings" filtrates as high as 1:200 were able to elicit the state of reactivity in normal rabbits, provided 5 to 25 reacting units were injected intravenously. In horse serum-sensitive rabbits strong reactions were obtained by skin-preparatory injections of dilutions

as high as 1:150 and failed with dilutions 1:200, provided 5 to 25 reacting units were injected intravenously.

Here again, horse serum sensitization did not enhance the skin-preparatory potency of the bacterial filtrates.

DISCUSSION

It seems that before any attempts can be made to study the nature of local skin reactivity to bacterial filtrates methods should be set forth for its quantitative measurements. Experiments described in

TABLE III

Titration of Skin-Preparatory Potency of Filtrates in Normal and Horse Serum-Sensitized Rabbits

Sensitization of rabbits prior to titrations	Intradermal injections		Intravenous injections		Results
	Material	Dilution	Material	No. of reacting units	
—	B.Ty T _L * 1916	1:200	B.Ty T _L 1916	5	2/1
—	" "	1:150	" "	25	2/1
—	" "	1:200	" "	5	2/1
—	B.Ty T _L 1938	1:200	B.Ty T _L 1938	25	2/1
Horse serum sensitization	" "	1:300	" "	5	0/3
	" "	1:60	" "	5	3/0
	" "	1:120	" "	5	2/1
	" "	1:200	" "	5	1±/2
" "	" "	1:300	" "	25	0/3

* Abbreviation B.Ty T_L designates "agar washings" filtrates of *B. typhosus*, Strain T_L cultures.

this paper demonstrate that the necessary estimations can be accomplished in a twofold manner: It is possible to determine the intensity of reactivity by studies on its duration. The duration depends upon the potency of preparations employed. It disappears within 48 hours with *B. typhosus* tryptic digest broth culture filtrates but persists for 72 hours with *B. typhosus* "agar washings" filtrates. Meningococcus "agar washings" filtrates yield a more protracted state of reactivity (up to 120 hours). Filtrates heated in the Arnold sterilizer for 20 minutes produce a state of reactivity of not longer than 24 hours duration. The various preparations were also titrated for the reacting

potency. It became obvious from these titrations that the duration of reactivity elicited by the various preparations is in direct relationship to their reacting potency.

It was previously reported that heating of the filtrates in the Arnold sterilizer at 100°C. does not destroy the phenomenon-producing power of the filtrates. It appears, however, from these experiments that the potency of the filtrates is at least partially decreased by exposure to heat, inasmuch as the duration of the reactivity elicited by filtrates heated at 100°C. for 20 minutes is considerably shorter than with unheated filtrates.

The reactivity can be also measured accurately by quantitative titrations of skin-preparatory factors against the constant intravenous dose of reacting factors. If a single skin site is prepared and 5 to 25 reacting units are injected intravenously, dilutions as high as 1:200 are able to prepare for severe hemorrhagic necrosis in a high percentage of rabbits. It is interesting to point out here that skin sites prepared with these dilutions undergo no gross inflammatory reactions prior to the intravenous injections. In most instances, it is impossible to detect the site of the preparatory injection with the naked eye. This appearance of the prepared skin site preceding the intravenous injection is in sharp contrast to the dramatic, hemorrhagic and necrotic lesion elicited a few hours after the intravenous injection. It is obvious, therefore, that the explanations of the mechanism of the phenomenon, offered by Menkin and Freund, as summations of either inflammatory or hemorrhagic effects of the two injections are untenable (6, 7).

Advantage was taken of the above experiments in order to add further evidence for differentiation of the phenomenon of local skin reactivity to bacterial filtrates from the Arthus phenomenon. As pointed out previously, there are clear-cut points of differentiation which are briefly as follows: short duration of local skin reactivity as proven again by the additional experiments presented in this paper; lack of passive transfer; short incubation period necessary for the elicitation of the reactivity, the necessity to give the test injection *via* the blood stream, specific neutralizations of the preparatory and reacting factors by immune sera; the inability to induce the

local reactivity by animal proteins; the non-specificity of the phenomenon, etc.

Recently the following observations were made. If rabbits are prepared by an injection of bacterial filtrates and 24 hours later a mixture of some animal protein with homologous antiserum is injected intravenously into the rabbits, there appears a severe hemorrhagic necrosis at the prepared skin site 4 hours after the intravenous injection of the antigen-antibody complexes. The interpretation of this experiment was that tissues exposed to the effect of certain soluble bacterial products become highly susceptible to humoral toxic principles resulting from antigen-antibody interaction. The humoral nature of these toxic principles was also clearly shown by experiments in passively sensitized rabbits. In these experiments the antigen was injected immediately or $\frac{1}{2}$ hour after the injection of the antibody.

In further work it was shown that the same reactions at the sites prepared by bacterial filtrates could be obtained by the intravenous or intradermal injection of the homologous antigen into rabbits previously sensitized. In these experiments one also dealt with active acquired cellular anaphylactic sensitization. It was important, therefore, to determine whether the tissue sensitization to animal proteins could influence the state of reactivity elicited by bacterial filtrates; and also whether the reactions of the Arthus phenomenon were enhanced by bacterial filtrates.

The data presented show that the state of protein hypersensitiveness does not influence to any appreciable degree the phenomenon of local skin reactivity to bacterial filtrates. In the experiments, either bacterial filtrates or horse serum were injected into normal and horse serum-sensitized rabbits prepared by intradermal injections of bacterial filtrates. The skin-preparatory potency of bacterial filtrates and the duration of the ensuing reactivity was not increased in horse serum-sensitized rabbits. Furthermore, the sensitization to horse serum was repeated several times and the duration of reactivity was studied after each sensitizing injection. In this manner, it was shown that repeated anaphylactic sensitization did not increase the reactivity induced by bacterial filtrates. If sensitization was continued long enough and the injection of horse serum alone was sufficient for elicit-

and *B. typhosus* "agar washings" filtrates previously heated in the Arnold sterilizer for 20 minutes.

Comparative titrations of the preparations employed demonstrate that the duration of reactivity is in direct relationship to the reacting potency.

It is also shown that the skin-preparatory potency of the filtrates and the duration of the ensuing local reactivity are not modified by cellular anaphylactic sensitization (Arthus phenomenon) to animal proteins.

The exposure of tissues to the effect of certain soluble bacterial factors induces a high susceptibility to humoral toxic principles resulting from intravascular antigen-antibody interaction.

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THE COLONY MORPHOLOGY OF TUBERCLE BACILLI

I. THE PRESENCE OF SMOOTH COLONIES IN STRAINS RECENTLY ISOLATED FROM SOURCES OTHER THAN SPUTUM

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PLATES 15 AND 16

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Variations in the colony morphology of pathogenic bacteria were observed many years ago, but it was not until the work of Arkwright (1, 3, 8), De Kruif (2, 4, 5), and Northrop and De Kruif (6, 7) that the significance of these variations began to be apparent. Since then, the chemo-immunological studies of Avery and his associates (9-14) have demonstrated the urgent need of a clearer understanding of the phenomena associated with differences in the colony form of bacteria. As result of studies on dissociation these facts are now evident: In the case of many pathogenic bacteria two principal colony types can be differentiated. Virulence is usually associated with the smooth or mucoid forms, whereas the rough or granular forms are of degraded virulence. Immunologic specificity is usually associated with the smooth form, the rough forms showing only group antigenic components. Since the smooth or mucoid forms are the normal ones, the S \rightarrow R change represents a variation known to be correlated with a loss of virulence and antigenic specificity.

Although differences in the colony form of tubercle bacilli have been recognized and associated with differences in virulence, non-granular smooth or mucoid colonies of human tubercle bacilli have not previously been observed.

Petroff and his associates were the first to study the dissociation of tubercle bacilli. They demonstrated two or more types of colonies in cultures of human (15, 18, 19, 27), attenuated bovine (BCG) (16, 17), virulent bovine (18, 19), and avian tubercle bacilli (18-20). Four colony types were differentiated and stabilized in the case of the avian bacilli. These were designated S, FS, R, and Ch.

The first two were highly virulent; the latter two relatively avirulent. The S and R forms closely resemble the corresponding forms of other bacterial species. In the case of the virulent bovine and the BCG strains the colony forms designated S also showed greater virulence than those designated R. The colonies of virulent bovine bacilli designated S resembled the corresponding S forms of other bacteria. Greater difficulty was encountered in studying human strains. In a preliminary report Petroff demonstrated (17, Plate I) rounded, shiny colonies of human tubercle bacilli freshly isolated from sputum. These colonies, when planted in fluid media, grew diffusely. The virulence was not reported. In later studies (18, 19, 27), however, the colonies designated as S were low, spreading, granular, with somewhat irregular borders (27, Figs. 3 and 4). They were more easily suspensible than the R forms and sharp differences in virulence, such as were observed with avian bacilli, were also reported. The authors state (27) that "The decrease in virulence of the H-37 R, when compared with the parent culture, is more notable than the increase in virulence of the S." Since the S forms of BCG, and especially of human bacilli, did not resemble the S forms of other bacteria, the designations S and R were applied, not to indicate the appearance of the colonies but rather to indicate the reaction to environment; namely, sensitive and resistant (27, footnote 2).

Further extensive investigations of dissociation of tubercle bacilli have been made by Reed and his associates. They adopted the terminology proposed by Petroff. In an early study (21), they observed S and R forms in a culture of presumably bovine origin. Neither was virulent. Soon, however, they extended their own and Petroff's observations to demonstrate (22) a correlation between colony structure, acid agglutination, and virulence. Again the S colonies illustrated in the plates were granular, although more virulent than the R forms (22, Plate I, Figs. 8, 9, 10). Rice (23) next used the complement fixation reaction with antigens from S and R variants, and antisera against each, to study the antigenic relationships. The S forms proved to be superior to the R as antigens. Antisera against S forms contained S specific antibodies, whereas R antisera did not. In subsequent reports (24, 25) these observations were extended and analyses made of the sera of tuberculous human beings using S and R antigens and correlating these results with the colony types of tubercle bacilli isolated from the sputum of each individual case (25).

In reviewing the work of these and other investigators who have studied the types of colonies occurring in cultures of human tubercle bacilli, it was apparent that the colonies designated S were in no wise similar in appearance to the mucoid or smooth forms of other pathogenic bacteria. (See Petroff (27) Figs. 3 and 4; and Reed and Rice (22) Plate I, Figs. 8, 9, 10.) However, these colonies do resemble the FS type of avian bacilli shown by Winn and Petroff (20, Plate XIX,

Figs. 4 and 5). The latter are possibly intermediate forms composed of both S and R elements. Such forms were recognized in pneumococcus cultures by Blake and Trask (26). It seemed likely, therefore, that the types of colonies of human tubercle bacilli heretofore referred to as S were in reality intermediate forms also. The present study was undertaken to determine whether smooth or mucoid colonies of human tubercle bacilli could be obtained, and if so, to identify some of the factors which influence colony morphology. Several strains of bacilli freshly isolated from sputum and from other human sources have been studied; and the influence on colony morphology of the acid or alkali routinely used in primary isolation has been investigated.

First Experiment

Material and Methods.—Tuberculous fluids, pus, or tissues, removed aseptically by aspiration or by biopsy from active cases of tuberculous arthritis, spondylitis, or meningitis, were received through the courtesy of Drs. Currier McEwen, of New York University Medical School, and of Dr. G. F. Kempf, of the Indianapolis City Hospital. The specimens were handled aseptically throughout. Each specimen, whether of fluid, pus, or tissue, was suspended in 15 to 45 cc. of sterile saline solution. The latter was then divided into three portions in sterile flasks. To each was added 7 drops of brom-thymol blue indicator. To one flask was then added an equal volume of 6 per cent (by weight) sulfuric acid and to a second flask an equal volume of 3 per cent (by weight) NaOH. After 15 to 20 minutes the contents of these two flasks were neutralized to the color reaction of the third (untreated) flask. The volume of the contents of the three flasks was then made the same by additions of sterile saline. Cultures were made on several solid media (slants) by pipetting 0.35 cc. of the suspension into the culture tube. The usual procedure of centrifuging the tuberculous material was eliminated so that growth on the primary cultures would be sufficiently sparse that individual colonies could be studied. The tubes were sealed with melted paraffin and incubated in a dark room at 37°C.

In some instances the tuberculous material was first inoculated into guinea pigs. These animals were killed with chloroform after 30 days, the spleen or femoral bone marrow removed aseptically, and cultures made in the manner described above.

For purposes of comparison with these freshly isolated, non-sputum strains of organisms, two additional strains of bacilli were isolated from the sputum of patients with rapidly advancing pulmonary tuberculosis. Both strains were cultivated indirectly from guinea pigs inoculated with untreated sputum, and one of the strains was also cultivated directly after digestion of separate portions with NaOH or H₂SO₄ as previously indicated.

All cultures were examined periodically with a binocular dissecting microscope and the final observations were made after 4 months' incubation.

Three general types of colonies were observed and each was present in cultures of every strain studied. They may be described as follows:

1. Rounded, glossy, hemispherical or sometimes broad and low, margin entire, no tendency to spread, moist and non-granular, easily suspended in saline. This type was designated S, indicating *smooth* (Figs. 1 and 2).

TABLE I

The Number of Smooth, Intermediate, and Rough Colonies of Human Tubercle Bacilli in Primary Cultures, and the Source and Manner of Isolation of Each Strain

Strain of bacilli	Source	Manner of isolation	Number of colonies*		
			Smooth	Intermedi-ate	Rough
Thompson	Psoas abscess	Direct	250	974	936
Kilty	Tuberculous wrist	Direct	100		
3104	Tuberculous knee	Direct	12	7	1
3103	Monkey spleen	Direct	940	258	2
3421	Spinal fluid	Indirect	10	275	120
3422	Spinal fluid	Indirect	813	249	64
MR	Tuberculous abscess spine	Indirect	1,955	8,834	2,311
Bell†	Sputum	Indirect	65	2,020	366
Burroughs	Sputum	Direct and indirect	61	945	444
Total, non-sputum strains.....			4,080	10,597	3,434
Total, sputum strains.....			126	2,965	810

* Growth recorded as scant (1 to 5 colonies), fair (6 to 20 colonies), good (21 to 100 colonies), and excellent (101 to 1,000 colonies) per culture. Estimates were made of the per cent of each type in each culture. The per cent multiplied by 5, 20, 100, or 1,000 gave the figure recorded. Example: 50 per cent, growth fair ($0.50 \times 20 = 10$). Any one figure in the table may be a composite for any number of cultures.

† These cultures not primary isolations but from a guinea pig inoculated with second cultural generation.

2. Broad, spreading, flat, finely granular, moist or dry, center often elevated, margin more or less irregular, rather easily suspended in saline. This type was designated I, indicating intermediate, and is similar to or identical with the type heretofore designated S (Fig. 3, B).

3. Coarsely granular, wrinkled, dry colonies of irregular contour,

rising sharply from the medium but adhering to its surface, suspensible in saline with difficulty. This form was designated R (Fig. 3, C).

The observations of the cultures made in primary isolations of seven strains of tubercle bacilli from sources other than sputum and of two strains from sputum are recorded in Tables I and II.

Table I presents the data on the source of each culture with an approximation of the whole number of colonies of each type present in all of the primary cultures of each strain. Table II is a composite of the data presented in Table I and shows the per cent of each type of colonies present in primary cultures of sputum and non-sputum strains. Tables I and II show that the incidence of smooth colonies in the cultures was far lower in the sputum strains than in the strains from other sources (with only one exception, Strain 3421). Strains Kilty

TABLE II

The Number and Per Cent of Various Types of Colonies of Tubercle Bacilli in Cultures Freshly Isolated from Sputum and from Other Sources. Summary of Data, Table I

Sources of organism	No. of strains	No. of cultures positive	No. of colonies Total*	Per cent of colonies		
				Smooth	Intermediate	Rough
Tissue other than sputum.....	7	45	18,111	22.5	58.5	18.9
Sputum.....	2	27	3,901	3.2	76.0	20.7

* See footnote (*) Table I.

3103 and 3422 (Fig. 2) showed the highest per cent of smooth colonies, namely, 100, 78, and 72 per cent. The figures for the latter two strains are more significant as they include a greater number of cultures. All of the strains considered in the tables, except Nos. 3421, 3422, and Burroughs, have been classified definitely as human strains by virulence tests in guinea pigs and rabbits. The differences noted in colony forms of sputum strains, as compared to non-sputum strains, cannot therefore be charged to differences in the type of organisms. It must be stated, however, that cultures predominantly smooth may be obtained on subcultivation of strains isolated from sputum (Fig. 4). It is therefore clear that freshly isolated tubercle bacilli from sources other than sputum do grow in colonies which conform in appearance to the S forms of other pathogenic bacteria.

The rôle of culture media and of methods of avoiding contaminations will be discussed in the following section.

Second Experiment

Material and Methods.—Each of eight human and eleven bovine strains of tubercle bacilli were inoculated intravenously into two rabbits and subcutaneously into two guinea pigs, a total of thirty-eight rabbits and thirty-eight guinea pigs. Cultures of the same age, grown on Corper's (28) egg yolk glycerine medium, or on Petroff's egg gentian violet medium were employed. The inoculating dose for each animal, both rabbits and guinea pigs, was 0.1 mg. of moist bacilli. Of the animals receiving human strains, one rabbit and one guinea pig were sacrificed at 30 and one at 60 days. Of the animals receiving bovine strains, one rabbit and one guinea pig were sacrificed at 30 days. The spleen or femoral bone marrow, or both, were removed aseptically and placed in sterile containers. Cultures were made as described in the previous experiment, from untreated, acid-treated, and alkali-treated tissue suspensions. The three portions of suspension were each seeded on at least three media which included (1) Petroff's gentian violet egg, (2) Corper's (28), and one or more of the following: (3) Petroff's medium with 0.25 per cent sodium taurocholate. (4) Egg yolk 30 per cent; glycerine 6 per cent; Fairchild's aminopeptone 2 per cent; water *q.s.* (5) Egg yolk 30 per cent; glycerine 6 per cent; calf spleen infusion 30 per cent; water *q.s.* (6) Petroff's medium without gentian violet. For the purpose of evaluating media and for studying individual colonies the inoculum was made quite dilute, as suggested by Corper and Cohn (28).

Cultures were examined periodically and finally, after 4 months' incubation, as in the previous experiment. The results described below as to types of colonies noted are the final results, as it was observed that the type of colony oftentimes changed with prolonged incubation. For instance, at 3 weeks some of the cultures showed a number of small colonies having the appearance of tiny droplets of cream. In a few instances some of these colonies became granular when incubated longer. Only the final appearance is considered therefore. Contaminated cultures were discarded and not considered in the computation of results. Needless to say, the greater number of these were in the cultures from tissues which were not treated with acid or alkali. A number of cultures showed no growth.

Two of the human strains used in this experiment were not included in the first experiment. They were (1) the H-37 strain isolated at Saranac in 1905 by Dr. E. R. Baldwin and obtained several years ago through the generosity of Dr. S. A. Petroff, and (2) a strain designated "Jamaica" isolated in November, 1933, from a Jamaican native and obtained through the courtesy of Dr. J. Freund, of Cornell University Medical School. The eleven bovine strains were all, save one, isolated by Dr. Theobald Smith, through whose munificence they were obtained. The other bovine strain was isolated by Ravenel and was supplied by Dr. E. R. Long, of the Phipps Institute. Each of the human strains produced progressive tubercu-

losis in guinea pigs and retrogressive lesions in rabbits. The H-37 and Jamaica were the least virulent of the human strains, though by no means avirulent. Five of the bovine strains proved to be of very low virulence. Two others were of moderate virulence and four were of high virulence. All of the virulent bovine strains produced progressive tuberculosis in both rabbits and guinea pigs.¹

The data on the cultures of human and bovine tubercle bacilli are included in Tables III and IV respectively. This experiment constitutes an animal passage and re-isolation of each strain. As in the first experiment, a large number of S colonies were present in the cultures, both of human and of bovine bacilli. The S colonies of human bacilli showed no striking difference from the S colonies of bovine bacilli (Figs. 5-7). Some of the cultures showed only a few S forms, while others were composed almost exclusively of them. Among the bovine cultures a few S colonies appeared in strains of intermediate and low virulence, whereas in the strains of greatest virulence the colonies were predominantly or wholly of the smooth type (Figs. 5-7). The same was true of the cultures of human strains, the smaller number of smooth colonies being found in the cultures of the two strains of least virulence; namely, Jamaica and H-37.

From the data included in Tables III and IV, it may be seen that, under the conditions of this experiment, medium Formula 2, namely Corper's, was definitely superior to the others, both as regards number of positive growths and the development of S colonies. In the light of previous investigations, this observation is not surprising as it was found (28) that egg white (contained in Formulae 1, 3, and 6) was of little value as a nutrient for tubercle bacilli and might even exert an inhibitory effect. Moreover, it was previously shown in a well controlled experiment (28) that the addition of peptone and meat infusion to egg yolk (as in Formulae 4 and 5) did not appreciably increase the nutrient qualities of the media. Undoubtedly if medium Formula 2 alone had been used, the per cent of cultures with smooth colonies would have been much higher.

Tables III and IV also indicate that the routine procedure of treating tuberculous material with acid or alkali to destroy contaminating organisms may have a marked effect on the type of colony which

¹ The details of this entire experiment will be reported in a subsequent communication.

develops. In the cultures of bovine strains from tissue treated with NaOH, 46 per cent of the positive cultures showed smooth colonies of the H_2SO_4 -treated, 54 per cent; and of the untreated, 63 per cent. In the cultures of human bacilli from tissue treated with NaOH, 3 per cent of the positive cultures showed smooth colonies; with H_2SO_4 , 35 per cent, and the untreated showed 61 per cent, differences which we believe to be highly significant. NaOH seemed in each instance to have a slightly greater tendency against the development of smooth colonies than did H_2SO_4 . In the elimination of contaminating organisms NaOH and H_2SO_4 appeared to be equally efficient.

From the above results it is evident that virulent cultures of tubercle bacilli of either bovine or human origin, after animal passage and re-isolation, show a considerable number of rounded, shiny, non-granular colonies, that these colonies are most numerous in cultures of greatest virulence, and that NaOH and H_2SO_4 treatment is not conducive to formation of S colonies. The significance of the presence of smooth colonies in cultures of low virulence remains to be determined.

Third Experiment

Material and Methods.—Strains Kilty and MR, used in Experiment 1, were used for additional studies. In the initial transplants only smooth or intermediate colonies were transferred. The subcultures grew predominantly as intermediate colonies, that is, low, flat, somewhat spreading, veiled, and finely granular, but there were also a moderate number of smooth colonies.

The first subculture of Strain MR was grown on egg yolk 30 per cent, glycerine 6 per cent, peptone 2 per cent (Formula 4). After 115 days' incubation, a portion of the growth was emulsified in saline and seeded on the surface of Corper's medium which had been adjusted by the addition of $N/1$ $NaHCO_3$ to pH 6.4, 6.7, 7.0, and 7.4. The original pH of two lots of this medium before adjusting was 6.15 and 6.2. These cultures were made in triplicate or quadruplicate. The inoculum was made quite dilute so that the individual colonies could be studied. The pH determinations were made by the glass electrode method through the courtesy of Dr. D. A. MacInnes, by Dr. L. G. Longsworth.

In attempting to adjust the pH to the desired values, $N/1$ NaOH was first added to two portions of the medium. It was noted that immediate coagulation of the medium occurred on introducing alkali, making small masses of solid in the surrounding liquid. It was obvious that the coagulated and fluid portions might have different pH values. The contents of these two flasks were then filtered through fine, sterile cheese-cloth to remove the coagulated material and the four lots of medium were adjusted to the desired value by adding small amounts of $N/1$

NaHCO_3 . After the appropriate adjustments had been made, the medium was inspissated in the usual manner in pint Blake bottles. After inspissation the pH of two lots of the medium was determined. It was found that the medium to which

TABLE V

Growth of Freshly Isolated Human Tubercle Bacilli in Blake Bottles of Corper's Medium Adjusted to Four Different pH Values

Culture Nos.	Corper's medium, 400 cc. lots					Observations on cultures
	NaOH N/1 added	pH before adding NaHCO_3	NaHCO_3 N/1 added	pH before inspissation	pH after inspissation	
MR 85, 86, 87, 88	No	6.15	2.5	6.4	6.5	Total growth abundant. Colonies 95 per cent S. 3 to 8 mm. in diameter, rounded, margin entire, shiny, moist, and non-granular. White
MR 89, 90, 91, 92	No	6.20	7.0	6.7	Not determined; probably about 7.0	Growth $\frac{1}{2}$ as abundant as above. Colonies 0.5 to 7 mm. in diameter. Three types of colonies. (1) Same as above 50 per cent; (2) small, dry, granular, wrinkled, irregular in contour (R) 20 per cent; (3) low, finely granular, veiled (intermediate) 30 per cent
MR 93, 94, 95, 96	2	6.50	14.5	7.0	8.0	$\frac{1}{2}$ as much growth as 89 to 92. Colonies 0.5 to 2 mm. in diameter. Of same appearance as (2) above (R) and (3) above (intermediate)
MR 97, 98, 99	4	7.07	8.0	7.3	Not determined, probably about 7.6	Sparse growth. Colonies 0.5 to 2 mm. in diameter. Small, dry, granular, irregular in outline as (2) above (R) and (3) above (intermediate)

had been added the greatest quantity of NaHCO_3 had changed in pH to 8.0. The pH 6.4 portion, to which had been added the least NaHCO_3 , had changed only slightly, namely, to pH 6.5 (Table V).

The pint Blake bottles were incubated on a slightly inclined plane, so that water of condensation would collect at one end. The medium was placed downward, so that the bacilli grew above the surface of the medium. The bottles were sealed with cellophane caps.

Marked differences were noted both in the amount of growth and the types of colonies which appeared in the four groups of cultures. Growth was luxuriant at 6.5, somewhat less so in the next series, and much less vigorous on the alkaline medium. At pH 6.5 the colonies were large, white, round in contour, flattened with convex surface, shiny, and non-granular (smooth). In the cultures adjusted to pH 6.7 (after inspissation about 7.0) the colonies were beautifully dissociated, about half being of the S form, as in the preceding group, and the remainder being dry, granular, and irregular in contour (intermediate and rough). In the cultures adjusted before inspissation to pH 7.0 and 7.3, the growth was much less luxuriant and only the intermediate and rough types of colonies were seen. For details see Table V.

The second serial subcultures of the freshly isolated Kilty (human) strain were grown on tubes of Petroff's gentian violet egg media. These cultures showed only finely granular, spreading (intermediate) colony forms. After 56 days' incubation dilute suspensions of the organisms were made and seeded on the surface of media in pint Blake bottles. The use of Blake bottles affords a large surface for growth so that many colonies may be studied, the moisture is better retained in the media than in Petri dishes, and the organisms are furnished a relatively large volume of air during growth. In this experiment 98 cultures were made on the following media formulae: (a) Corper's egg yolk-glycerine medium. (b) Medium A plus 2 per cent Fairchild's aminopeptone. (c) Medium A plus 3 cc. N/1 NaHCO_3 per liter. The bottles were sealed with cellophane and incubated in the manner described for the cultures of Strain MR.

Sharp and striking differences in the colony morphology of organisms grown on the three media were noted. On standard Corper formula (twenty-two cultures) the growth was abundant and consisted of three principal types of colonies, as described in Experiment 1. It must be noted, however, that the smooth colonies were all small, rounded, and none exceeded 4 mm. in diameter. These smooth colonies constituted not more than 15 per cent of the total, the remaining 85 per cent or more being of the intermediate and rough forms (see Figs. 8 and 9). In the cultures (ten bottles) on Corper's formula, to which had been added 2 per cent Fairchild's peptone, the smooth colonies constituted about 65 per cent of the growth. These smooth colonies ranged in size from 2 to 7 mm. in diameter, were moist and shining, and showed a pale yellow-green color, the significance of

which is, as yet, undetermined. The remaining 35 per cent of colonies were of the intermediate and rough forms. Sixty-five cultures on Corper's formula to which was added sodium bicarbonate, also showed about 65 per cent smooth and 35 per cent intermediate and rough colonies. In these, however, the colonies were non-pigmented. The smooth colonies ranged up to 8 mm. in diameter and were very moist. At 30 days some were so mucoid that they would flow slowly across the medium when the surface of the latter was sharply inclined. On aging (and drying) these colonies lost some of their luster and showed a very fine structure (Figs. 10 and 11). These smooth colonies could be suspended uniformly and with ease in saline.

Additional experiments are in progress to determine (1) whether various strains and types of tubercle bacilli show an optimum pH for development of S colonies; (2) the significance of S colonies in virulent and less virulent strains; and (3) the antigenic relationships of S and other colony variants.

DISCUSSION

The observations of this study differ from those made by previous workers investigating phenomena of dissociation in cultures of tubercle bacilli, in that colony forms have been recognized which resemble in appearance the smooth forms of other bacteria. The smooth colonies were first obtained in cultures freshly isolated from sources other than sputum. They were most numerous when the inoculum for the culture had not been treated with acid or alkali. Preliminary observations indicate that the pH of the media on which freshly isolated non-sputum strains of tubercle bacilli are grown may be an important factor in determining the form of colonies which develop. Certain media seem also to be more favorable for the formation of smooth colonies than do others. In regard to the apparent influence of peptone toward the formation of smooth colonies (third experiment), it must be noted that this is possibly an effect only of pH. The peptone solution added to the medium is alkaline and the effect of weak alkali in the same basic medium was similar to that of peptone.

Parallel virulence tests with smooth, intermediate, and rough variants have not been completed. Nevertheless, it was noted that strains of high virulence show a high per cent of smooth colonies,

and that in strains of less virulence other types of colonies predominate. This observation is similar to that of Dawson and Olmstead (29) in the case of streptococci. It must be added, however, that all strains showing smooth colonies are not necessarily of high virulence. It will be shown elsewhere that in the case of two non-chromogenic strains of avian tubercle bacilli, each of which produces only smooth colonies, one is virulent while the other is relatively avirulent.

The full significance of such observations as those described in the present study must be determined by further investigation. However, it is believed that the normal colony form for fully virulent tubercle bacilli of mammalian origin, under the most favorable circumstances, is smooth. Moreover, it is believed that environment unfavorable to the organisms, whether *in vivo* or *in vitro*, tends toward the development of finely and then more coarsely granular colonies, with attendant changes in virulence and antigenic structure. Again these observations and considerations differ from those of others in that smooth colonies are recognized which have heretofore not been observed, or have been regarded as of little significance.²

SUMMARY

1. Smooth, round, shiny, non-granular, and non-spreading colonies have been observed in cultures of virulent tubercle bacilli freshly isolated from eight human sources other than sputum. The classification of six of these strains as of human type was established by inoculation into rabbits and guinea pigs.

2. The 3 per cent NaOH or 6 per cent H₂SO₄ frequently used in the isolation of tubercle bacilli are definitely unfavorable to the development of smooth colonies.

3. It was observed that smooth colonies are produced in greater number when the pH of the medium (Corper's egg yolk-glycerine) is adjusted to a point near to but slightly on the acid side of neutral.

² While the present work was in progress a preliminary communication by Alexander (30) appeared, in which the author obtained smooth, convex, glistening colonies of human tubercle bacilli on Bordet-Gengou medium modified by the addition of 0.0004 per cent ferric chloride. The writer mentioned that there were differences in the lesions produced by the R and S forms, and that the S forms were somewhat more virulent. Details of this work have not yet appeared.

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EXPLANATION OF PLATES

PLATE 15

FIG. 1. Smooth colonies of human tubercle bacilli: Strain MR isolated from a patient with a tuberculous abscess of the spine. Age of culture 219 days. $\times 8.5$.

FIG. 2. Smooth colonies of tubercle bacilli: Strain 3422 isolated from the spinal fluid of an adult with tuberculous meningitis. Age of culture 91 days. $\times 8.5$.

FIG. 3. A, smooth; B, intermediate; and C, rough colonies of a strain (Burroughs) freshly isolated from sputum. Age of culture 72 days. $\times 8.5$.

FIG. 4. Smooth colonies of a strain of human tubercle bacilli recently isolated from sputum. Second subculture after animal passage. Corper's medium. Age of culture 101 days. $\times 8.5$.

FIG. 5. Smooth colonies of bovine tubercle bacilli: Strain 36 re-isolated from the spleen of a rabbit after intravenous inoculation. Age of culture 119 days. $\times 8.5$.

FIG. 6. Smooth colonies of bovine tubercle bacilli: Strain 39 re-isolated from the spleen of a rabbit after intravenous inoculation. Age of culture 119 days. $\times 8.5$.

PLATE 16

FIG. 7. Smooth colonies of bovine tubercle bacilli: Strain 40 re-isolated from the spleen of a rabbit after intravenous inoculation. Age of culture 119 days. $\times 8.5$.

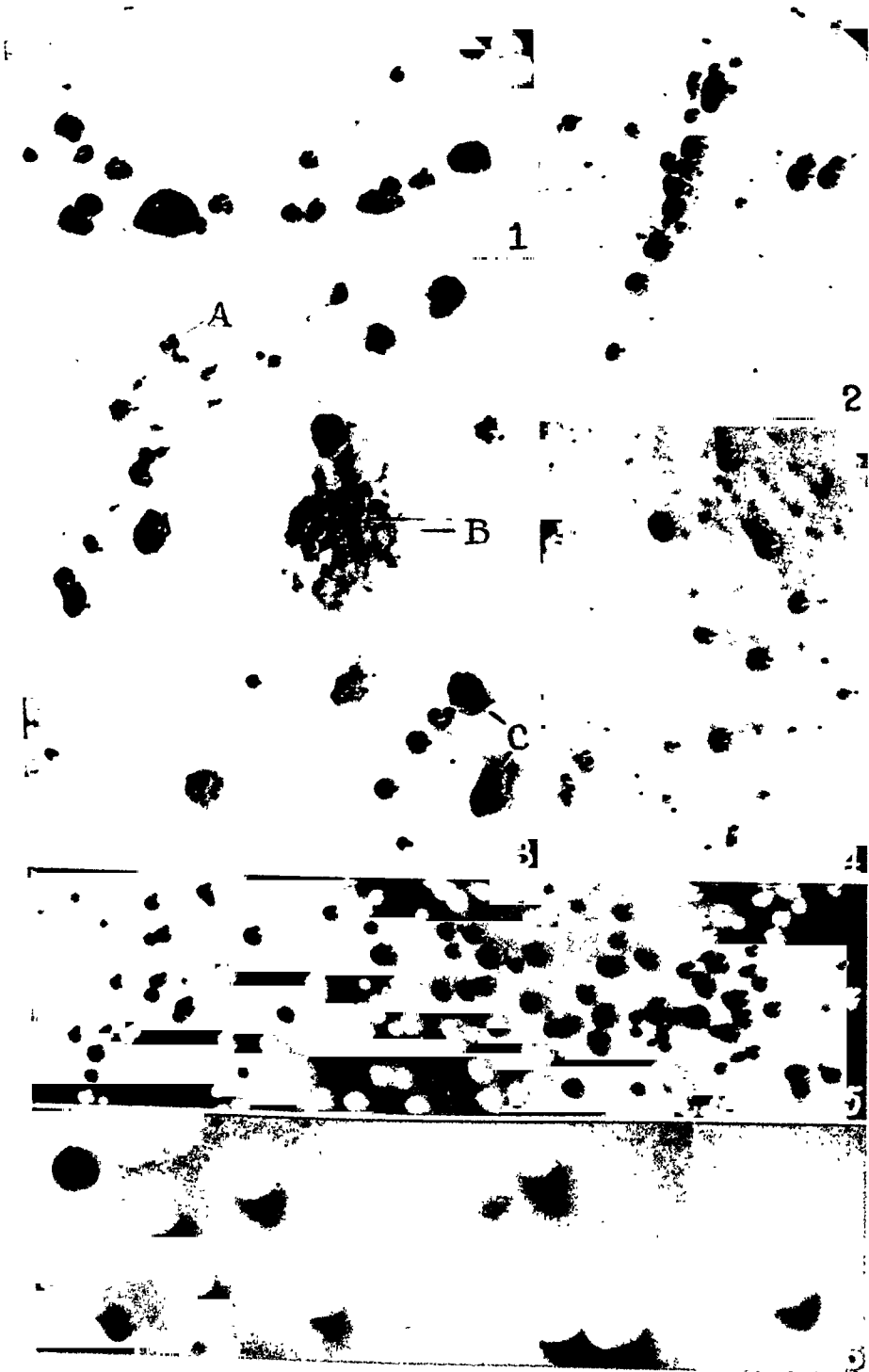
FIG. 8. Growth of human tubercle bacillus, Strain Kilty, on Corper's medium. Colonies granular. Age of culture 129 days. $\times 8.5$.

FIG. 9. Another field from the same culture as Fig. 8, showing *A*, intermediate, and *B*, rough colonies. $\times 8.5$.

FIG. 10. Smooth colonies, Kilty (human) strain, on Corper's medium plus 3 cc. $\text{N}/1 \text{ NaHCO}_3$ per liter. Seeded from same suspension as cultures in Figs. 8 and 9. Age 129 days. $\times 5$.

FIG. 11. Another field, same culture as in Fig. 10. $\times 8.5$.

In order to make photographs of these cultures they were removed from the containers and placed in Petri dishes, the covers of which were replaced by plate glass. Photographed on horizontal stage with 75 mm. lens, diaphragm 8 mm. Wratten and Wainwright, M plate. Pointolite with aspheric condenser.



EXPERIMENTAL STUDIES ON ENCEPHALITIS

II. THE SPECIFIC VIRUS CHARACTER OF THE INFECTIOUS AGENT FROM CASES OF ST. LOUIS AND KANSAS CITY ENCEPHALITIS, 1933

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(Received for publication, December 18, 1934)

In previous papers (1, 2) we have described the successful transmission of encephalitis to mice by injecting them with brain tissue from fatal cases from the St. Louis and Kansas City epidemic of 1933. The infective material proved free from ordinary microorganisms and remained infective for about 32 days when preserved in 50 per cent glycerin (2). Further properties now to be described indicate that the agent is a filtrable virus probably different from any hitherto known.

Filtrability

We have reported that the agent readily traverses Berkefeld V and N candles (1). The following test demonstrates its passage through Seitz pads in high dilution without appreciable loss.

Experiment 1.—Brains from two mice prostrate 4 days after an intracerebral injection of infective mouse brain, Strain 3, were removed, weighed, ground with alundum, and diluted one part by weight to ten parts by volume with Bauer's fluid composed of equal parts of distilled water, pneumococcus broth, pH 7.8, and sterile ascitic fluid (3). This emulsion was centrifuged at low speed for 10 minutes and 1 cc. of supernatant fluid was combined with further diluent to secure final brain virus concentrations of 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} gm. 3 cc. portions of each dilution were then filtered simultaneously at 30 pounds pressure through Seitz pads which had been satisfied previously with 10 cc. of pneumococcus broth at 15 pounds pressure. The filtered and unfiltered portions of each dilution were then injected intracerebrally in 0.03 cc. amounts into four Swiss mice. The duration of life of the injected animals was noted in days.

The results of the test are shown in Table I. Both unfiltered and filtered specimens in dilutions of 10^{-4} to 10^{-7} inclusive killed all mice.

In the 10^{-7} dilution the unfiltered specimen killed all four mice after a considerable delay, while the filtered specimen killed two of four mice. All mice receiving unfiltered and filtered specimens in dilution of 10^{-8} survived. This close approximation of the titer of the filtered and unfiltered mouse brain extract in high dilution has been observed repeatedly.

To determine the minimum average pore size of filters permitting passage of the virus, tests were made with graded collodion membranes according to the general method of Elford (4). With the help of Dr. J. H. Bauer, who prepared and calibrated the membranes and filtered the suspensions of virus, nine tests with twenty-five membranes of different porosities were made. The results, tabulated elsewhere, show that membranes with average pore diameters of 66 μ

TABLE I

Filtration of Small Quantities of Encephalitis Virus, Strain 3, through Seitz Pads

Virus Strain 3	Dose 0.03 cc. intracerebrally in dilutions				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	Duration of life				
	days	days	days	days	days
Unfiltered.....	5, 5, 6, 6	5, 5, 5, 6	6, 6, 8, 8	4, 8, 8, 11	S, S, S, S
Filtered.....	5, 6, 8, 8	5, 6, 6, 6	6, 8, 8, 8	6, 8, S, S	S, S, S, S

S = survived and well, 21 days.

or greater permit passage of the virus, while those with 62.5 μ average pore diameter or less do not permit passage of the virus (5). These findings have since been confirmed by Elford and Perdrau (6). The technique and protocol of one of the nine tests are given below.

Experiment 2, Test 7, Jan. 18, 1934.—Five brains from mice prostrate 4 days after an intracerebral injection of virus, Strain 3, were removed, emulsified with abrasive, and suspended in Bauer's diluent consisting of ascitic fluid, 35 cc., hormone broth, 35 cc., and distilled water, 30 cc. The suspension was centrifuged 30 minutes at 3,000 R.P.M. The supernatant fluid was filtered first through a Seitz pad and then through a collodion membrane, No. 166, with average pore diameter of 260 μ . Eight portions of this stock filtrate were then each passed through one of a graded series of membranes with average pore diameters ranging from 90.8 to 54.4 μ under a pressure of 100 cm. of mercury. The membrane filtrates together with the stock filtrate further diluted to 10^{-3} , 10^{-4} , 10^{-5} , and

10^{-6} were each injected intracerebrally in 0.03 cc. quantities into six Swiss and six Rockland mice. The mice were observed 21 days and the survival times recorded in days.

The result of this titration is recorded in Table II. The stock filtrate in each of the dilutions 10^{-3} , 10^{-4} , and 10^{-5} killed eleven of twelve mice and killed three of twelve mice in the 10^{-6} dilution. The stock filtrate put through membranes with average pore diameters of 90.8, 82.6, 71.2, 67.0, and 66.1 μ inclusive killed all mice, but put through membranes with average pore diameters of 57, 54.8, and 51.8

TABLE II

Filtration of Encephalitis Virus, Strain 3, through Graded Collodion Membranes, Experiment 7, Jan. 8, 1934

No. of membrane	Average pore diameter	Duration of life	
		Swiss mice	Rockland mice
	μ	days	days
166	260	5, 5, 6, 6, 6, 6 (dilution 10^{-3})	5, 5, 6, 7, 8, S (dilution 10^{-3})
166	260	6, 6, 6, 6, 7, S (" 10^{-4})	6, 6, 6, 7, 7, 9 (" 10^{-4})
166	260	6, 6, 7, 7, 7, 8 (" 10^{-4})	6, 6, 7, 7, 8, S (" 10^{-4})
166	260	7, 7, S, S, S, S (" 10^{-4})	8, S, S, S, S, S (" 10^{-4})
87	90.8	4, 5, 5, 5, 6, 6	5, 5, 6, 6, 6, 7
86	82.6	4, 5, 5, 5, 5, 6	5, 5, 5, 6, 6, 7
89	71.2	5, 5, 6, 6, 7, 7	6, 6, 7, 7, 7, 8
77	67.0	5, 5, 5, 5, 6, 6	5, 5, 5, 6, 6, 6
139	66.1	6, 6, 6, 6, 6, 7	5, 6, 6, 7, 7, 7
91	57	All remained well	All remained well
70	54.8	" " "	" " "
79	51.8	" " "	" " "

S = survived and well, 21 days.

μ failed to kill. This one experiment indicates that virus traverses membranes with average pore size of 66 μ or greater and is held back by membranes with 57 μ average pore size or less.

Susceptibility of Animals to Encephalitis Virus

Mice.—Mice are highly susceptible to the virus given intracerebrally as shown by the following titrations.

Experiment 3.—Brains of two mice prostrate 4 days after intracerebral injection of mouse brain virus, Strain 3, were removed, weighed, emulsified with abrasive,

In the 10^{-7} dilution the unfiltered specimen killed all four mice after a considerable delay, while the filtered specimen killed two of four mice. All mice receiving unfiltered and filtered specimens in dilution of 10^{-8} survived. This close approximation of the titer of the filtered and unfiltered mouse brain extract in high dilution has been observed repeatedly.

To determine the minimum average pore size of filters permitting passage of the virus, tests were made with graded collodion membranes according to the general method of Elford (4). With the help of Dr. J. H. Bauer, who prepared and calibrated the membranes and filtered the suspensions of virus, nine tests with twenty-five membranes of different porosities were made. The results, tabulated elsewhere, show that membranes with average pore diameters of 66 μ

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	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	Duration of life				
	days	days	days	days	days
Unfiltered.....	5, 5, 6, 6	5, 5, 5, 6	6, 6, 8, 8	4, 8, 8, 11	S, S, S, S
Filtered.....	5, 6, 8, 8	5, 6, 6, 6	6, 8, 8, 8	6, 8, S, S	S, S, S, S

S = survived and well, 21 days.

or greater permit passage of the virus, while those with 62.5 μ average pore diameter or less do not permit passage of the virus (5). These findings have since been confirmed by Elford and Perdrau (6). The technique and protocol of one of the nine tests are given below.

Experiment 2, Test 7, Jan. 18, 1934.—Five brains from mice prostrate 4 days after an intracerebral injection of virus, Strain 3, were removed, emulsified with abrasive, and suspended in Bauer's diluent consisting of ascitic fluid, 35 cc., hormone broth, 35 cc., and distilled water, 30 cc. The suspension was centrifuged 30 minutes at 3,000 R.P.M. The supernatant fluid was filtered first through a Seitz pad and then through a collodion membrane, No. 166, with average pore diameter of 260 μ . Eight portions of this stock filtrate were then each passed through one of a graded series of membranes with average pore diameters ranging from 90.8 to 54.4 μ under a pressure of 100 cm. of mercury. The membrane filtrates together with the stock filtrate further diluted to 10^{-3} , 10^{-4} , 10^{-5} , and

10^{-6} were each injected intracerebrally in 0.03 cc. quantities into six Swiss and six Rockland mice. The mice were observed 21 days and the survival times recorded in days.

The result of this titration is recorded in Table II. The stock filtrate in each of the dilutions 10^{-2} , 10^{-4} , and 10^{-5} killed eleven of twelve mice and killed three of twelve mice in the 10^{-6} dilution. The stock filtrate put through membranes with average pore diameters of 90.8, 82.6, 71.2, 67.0, and 66.1 μ inclusive killed all mice, but put through membranes with average pore diameters of 57, 54.8, and 51.8

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No. of membrane	Average pore diameter	Duration of life	
		Swiss mice	Rockland mice
	μ	days	days
166	260	5, 5, 6, 6, 6, 6 (dilution 10^{-2})	5, 5, 6, 7, 8, S (dilution 10^{-2})
166	260	6, 6, 6, 6, 7, S (" 10^{-4})	6, 6, 6, 7, 7, 9 (" 10^{-4})
166	260	6, 6, 7, 7, 7, 8 (" 10^{-4})	6, 6, 7, 7, 8, S (" 10^{-4})
166	260	7, 7, S, S, S, S (" 10^{-4})	8, S, S, S, S, S (" 10^{-4})
87	90.8	4, 5, 5, 5, 6, 6	5, 5, 6, 6, 6, 7
86	82.6	4, 5, 5, 5, 5, 6	5, 5, 5, 6, 6, 7
89	71.2	5, 5, 6, 6, 7, 7	6, 6, 7, 7, 7, 8
77	67.0	5, 5, 5, 5, 6, 6	5, 5, 5, 6, 6, 6
139	66.1	6, 6, 6, 6, 6, 7	5, 6, 6, 7, 7, 7
91	57	All remained well	All remained well
70	54.8	" " "	" " "
79	51.8	" " "	" " "

S = survived and well, 21 days.

μ failed to kill. This one experiment indicates that virus traverses membranes with average pore size of 66 μ or greater and is held back by membranes with 57 μ average pore size or less.

Susceptibility of Animals to Encephalitis Virus

Mice.—Mice are highly susceptible to the virus given intracerebrally as shown by the following titrations.

Experiment 3.—Brains of two mice prostrate 4 days after intracerebral injection of mouse brain virus, Strain 3, were removed, weighed, emulsified with attrative

diluted 1 to 10 with pneumococcus broth or ascitic fluid mixture, centrifuged 10 minutes at low speed and further diluted by serial tenfold dilutions. 0.03 cc. of each dilution was given intracerebrally to Swiss mice. The mice were observed 21 days and the duration of life noted. The results of two titrations are given in Table III.

The tests show (Table III) that the agent given intracerebrally in the manner described above is active in dilutions of 3×10^{-8} gm. of infected mouse brain and occasionally in dilutions of 3×10^{-9} gm.

The agent in stock mice is somewhat less active and regular in its effects and in R. I. S. mice (7), still less virulent (Table III).

Intranasal instillations of virus into Swiss, white-face, and R. I. R. mice give rise to characteristic fatal encephalitis (1). Following an incubation period of 5 to 6 days, hyperesthesia and tremors appear, followed by convulsions, prostration, and death in 7 to 10 days. Table IV illustrates the degree of activity of virus prepared in the manner described in Experiment 3 and instilled in 0.03 cc. amounts into the nares of Swiss mice.

The virus is less active when instilled intranasally into stock mice and still less virulent in R. I. S. mice (Table IV).

Intranasally infected mice show lesions similar to those in mice injected intracerebrally (1). Perivascular and focal accumulations of round cells are scattered throughout the brain and cord, and the characteristic necroses of the pyramidal cells of the piriform lobe and Ammon's horn are conspicuous. The earliest lesions are found 3 days after nasal instillation of virus in the olfactory bulbs beneath the pia. Round cells are scattered and collected in foci about the blood vessels relatively near the pia. By the 4th day the brain is involved in the piriform lobe beneath the pia. On the 5th day, Ammon's horn is affected. This orderly progression of lesions will be described more fully in a subsequent paper.

The injection of mice with the agent intraperitoneally, subcutaneously, and intravenously is without effect unless at least 100,000 intracerebral lethal doses are employed. If 0.1 gm. of brain virus is injected intraperitoneally into ten or more mice, 10 to 30 per cent may die of a typical encephalitis. Even this overwhelming dose usually fails to affect mice if it is administered subcutaneously.

Macacus rhesus Monkeys.—We have reported that *Macacus rhesus*

TABLE III

Infectivity of Encephalitis Virus, Strain 3, Injected Intracerebrally into Swiss, R. I. Stock, and R. I. S. Mice

Date	Strain of mice	No. mice per dilution	Dose 0.01 cc. intracerebrally in dilutions										
			10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹		
			Duration of life										
Oct. 8 Nov. 23	Swiss	4	days	days	days	days	days	days	days	days	days	days	days
	"	3	—	—	—	—	5, 5, 5, 6	5, 5, 5, 5	5, 6, 7, 9	7, 7, 8, 9	S, S, S, S	S, S, S, S	S, S, S, S
	Stock	6	3, 4, 4	4, 4, 5	4, 5, 5	4, 5, 7	5, 5, 7	4, 7, 7	7, 7, 7	S, S, S	S, S, S	S, S, S	S, S, S
	R. I. S.	3	4, 4, 7	4, 4, 4	4, 4, 4	4, 5, 5	5, 5, 5	5, 5, 5	7, 10, 11	7, 10, 11, S, S, S	S, S, S	S, S, S	S, S, S
			7, 7, 7	7, 7, 10	7, 7, 11	7, 10, 12	7, 10, 12	7, 7, 14	10, S, S	10, S, S	S, S, S	S, S, S	S, S, S
			10, 12, 13	10, 12, S	5, 12, S	—	—	—	—	—	—	—	—

S = survived and well, 21 days.

S = survived and well, 21 days.

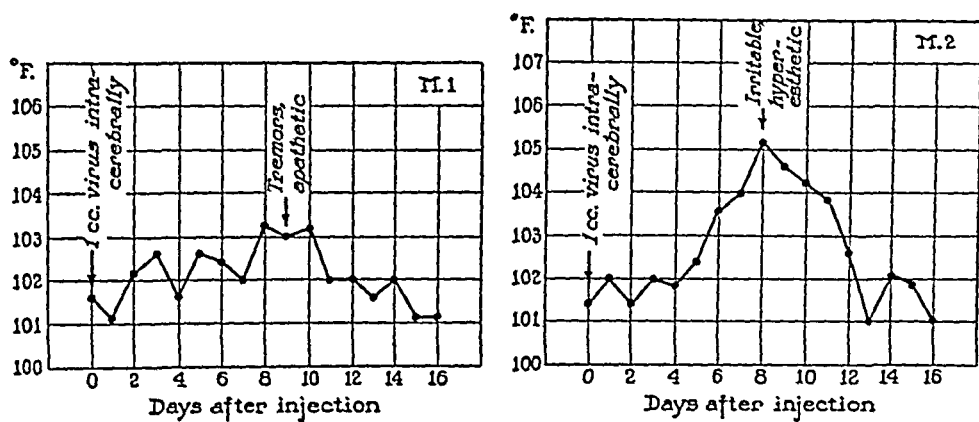
TABLE IV

Infectivity of Encephalitis Virus, Strain 3, Injected Intranasally into Swiss, R. I. Stock, and R. I. S. Mice

Date	Strain of mice	No. mice per dilution	Dose 0.03 cc. intranasally in dilutions					
			10 ⁻¹	10 ⁻²	2 × 10 ⁻³	10 ⁻³	6 × 10 ⁻⁴	2 × 10 ⁻⁴
Duration of life								
1914	Oct. 8	Swiss	6	days	days	days	days	days
	Nov. 21	"	4	7, 7, 7, 7	7, 7, 8, 8, 8, 9	8, 8, 8, 8, 8, 9	7, 7, 8, S, S, S	7, 7, 8, S, S, S
	" 23	Stock	6	7, 7, 7, 11, 12, 12	7, 10, 10, 10	7, 11, 13, S	7, 7, 11, S	S, S, S, S, S
	" 23	R. I. S.	4	7, 10, 10, 11	7, 7, 12, S, S, S	S, S, S, S, S	S, S, S, S, S	S, S, S, S, S
S ~ survived and well, 21 days.								

S = survived and well, 21 days.

monkeys given 1 cc. of a 10 per cent suspension of mouse brain virus intracerebrally showed elevations of temperature in 7 to 9 days, lasting 3 to 5 days, and followed by hyperirritability or apathy. Lesions appeared only in the central nervous system and consisted of scattered accumulations of round cells in perivascular spaces and in foci adjoining affected nerve cells (1). The meninges often contained rather prominent accumulations of round cells. Unlike the mice, monkeys showed no changes in the pyramidal cells of piriform and hippocampal lobes; they did present, however, very occasional injured nerve cells scattered irregularly in cortex, stem, and cord.



TEXT-FIG. 1. Temperature records of *Macacus rhesus* monkeys given 1 cc. of mouse brain virus intracerebrally.

Of six monkeys receiving mouse brain virus, all showed elevated temperatures after 7 to 9 days. Four of six showed tremors, irritability, and weakness. None succumbed but three continued ill for 6 weeks. Protocols of two of these animals are given below and the difficulty of establishing the virus in monkeys is described.

Experiment 4.—Sept. 18, 1933, Monkeys 1 and 2 inoculated intracerebrally with 1 cc. of mouse brain virus, Strain 4. Daily temperature records are plotted in Text-fig. 1. Monkey 1 showed slight elevation in temperature on the 8th day, and tremors of extremities, head, and back, and apathy on the 9th day. On the 10th day, the tremors continued and clonic convulsive movements of the hands and feet were prominent, together with extreme apathy, weakness when roused, and spasticity of the left leg. On the 11th day the signs were unchanged. Spinal fluid showed 92 cells and a positive Pandy. The animal was much improved on

the 13th day. On the 14th day the animal was sacrificed. Its cortex proved infective for mice; its blood and spinal fluid non-infective. Blood taken on the 10th day was later shown (Experiment 5) to contain specific protective properties against the virus.

Monkey 2 showed progressively increasing temperature after the 5th day, reaching a maximum on the 8th day. At this time it was irritable and hyperesthetic. On the 9th day these signs had increased and the left arm was ataxic. The next day these signs were at a maximum. The animal was extremely irritable, quite spastic, with convulsive movements. On the 11th day the spinal fluid was found to contain 43 cells and to show a positive Pandy test. By the 13th day the animal was still exceedingly irritable and wild in its movements. By the 20th day the animal was much improved and, a week later, seemed well.

Oct. 12, 1933. Monkey 5, given an intracerebral injection of 1 cc. of mouse brain virus, Strain 3, diluted 1 to 20, was sacrificed 12 days later at the height of fever and signs of encephalitis. Blood and spinal fluid injected into mice were ineffective; cortex injected into mice gave rise to characteristic fatal encephalitis. Cortex diluted 1 to 10 was also injected intracerebrally in 1 cc. amounts into two monkeys, Nos. 7 and 8. Both of these showed elevation of temperature on the 9th and 10th days, with tremors. No. 7 continued weak, had convulsions, and 6 weeks later was well. No. 8 was sacrificed on the 11th day. Its cortex proved infective in mice; its blood and spinal fluid negative. Intracerebral injections of cortex into three monkeys provoked a slight rise in temperature in two, with some irritability, weakness, and spasmodic movements. A single third passage experiment was carried out in two monkeys. Both showed slight elevations in temperature and doubtful signs of encephalitis. No further tests were made at this time.

Later, Nov. 15, 1934, each of the five strains of mouse brain virus was injected intracerebrally into a *Macacus rhesus* monkey with results similar to those described above.

Rabbits.—10 per cent suspensions of mouse brain virus given intracerebrally, intraocularly over the surface of the scarified cornea, intradermally, and intraperitoneally to young and adult rabbits provoked no demonstrable reactions.

Guinea Pigs.—Mouse brain virus in 10 per cent emulsions injected intracerebrally and into the pads of young and adult guinea pigs was innocuous.

Rats.—Rats given 10 per cent suspensions of mouse brain virus intracerebrally, intranasally, and intraperitoneally remained well.

Sheep.—One sheep given 2 cc. of a 10 per cent mouse brain virus suspension intracerebrally remained well.

Protective Action of Serum from Infected Monkeys

Sera from certain infected monkeys showed specific protective properties against the virus. Some of the tested monkeys had received a single intracerebral injection of mouse brain virus; others one or two additional intraperitoneal injections of 5 cc. of 1 to 10 virus 4 to 6 weeks later. The protection tests were carried out as follows:

Two brains from mice prostrate 4 days after an intracerebral injection of mouse brain virus, Strain 3, were removed, weighed, emulsified with abrasive, and taken up in pneumococcus broth pH 7.8 in a dilution of one part brain in fifty parts fluid. This material was then centrifuged at low speed and 1 cc. of supernatant diluted further. 0.3 cc. of each dilution was then added to 0.3 cc. of the test serum. The mixtures were placed in the incubator for 2 hours, left 1 hour at room temperature, drawn up in 0.25 cc. tuberculin syringes with 26 gauge needles, and injected intracerebrally in 0.03 cc. volume into four to six Swiss or R.I.R. mice. The duration of life was recorded in days.

The results of several tests are shown in Table V. Serum from Monkey 1, drawn 10 days after the primary intracerebral injection, when the animal showed elevated temperature and definite symptoms, gave definite protection; that is, all but one animal receiving the monkey serum plus the three highest dilutions of virus survived, as contrasted with the death of all but one receiving corresponding dilutions plus control serum. On the contrary, serum from Monkey 8, drawn under similar conditions, did not protect. Again, serum from Monkey 2, drawn several weeks after the initial injection and 2 weeks after a series of three intraperitoneal injections, showed strong protective properties which appeared to decrease with the age of the specimen. A second specimen, obtained after further intraperitoneal injection, showed similar strong protective properties which disappeared after 4 months. No preservative was used: specimens were kept sealed in the ice box.

Serological Uniformity of Strains

The strains of virus from four St. Louis cases and one Kansas City case proved alike in their effects in mice and *Macacus rhesus* monkeys. Moreover, two additional strains which Muckenfuss, Armstrong, and McCordock obtained by injecting brain tissue from St. Louis cases into *Macacus rhesus* monkeys and later from infected monkeys into

TABLE V
Protective Effect of Immune Monkey Sera on Homologous Encephalitis Virus

Experimental Data on Homologous Encephalitis Virus

Test No.	Date	History of test serum	Dose 0.03 cc. intracerebrally in dilutions				Result
			10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
			Duration of life				
			days	days	days	days	days
8	Nov. 2	No. 1 fresh from M. 1, 10 days after injection	5, 6, 6, 8	9, S, S, S	S, S, S, S	S, S, S, S	Positive
	" 2	Human non-contact	4, 4, 5, 6	4, 5, 6, 6	5, 5, 6, 7	5, 7, 7, S	
12	" 16	No. 2 fresh from M. 2, 10 days after injection	5, 5, 5, 6	5, 5, 5, 6	6, 7, 7, 8	6, 7, 8, 10	Negative
	" 16	Human non-contact	4, 5, 5, 6	5, 5, 6, 6	5, 6, 6, 6	5, 5, 7, S	
18	Jan. 17	No. 3 fresh from M. 2, 14 days after serial injection	—	S, S, S, S, S, S	S, S, S, S, S, S	—	Positive
	" 17	Human non-contact	—	5, 6, 6, 6, 6, 7	6, 6, 6, 6, 7, 7	—	
19	Feb. 8	No. 3, 5 wks. at 4°C.	—	7, 7, S, S, S, S	7, 7, S, S, S, S	—	Positive
	" 8	Human non-contact	—	5, 5, 5, 5, 5, 6	5, 5, 6, 6, 6, 7	—	
24	Mar. 19	No. 3, 10 wks. at 4°C.	—	5, 6, 7, 7, 8, S	S, S, S, S, S, S	—	Positive
	" 19	Human non-contact	—	5, 5, 5, 5, 5, 5	5, 5, 5, 5, 5, 6	—	
26	Apr. 11	No. 3, 13 wks. at 4°C.	—	5, 5, 5, 5, 5, 7	5, 7, 7, 7, S, S	—	Negative
	" 11	Human non-contact	—	4, 4, 5, 5, 5, 6	5, 5, 5, 6, 6, 7	—	
30	June 5	No. 4 fresh from M. 2, 14 days after repeated injection	—	S, S, S, S, S, S	S, S, S, S, S, S	—	Positive
	" 5	Human non-contact	—	6, 6, 6, 7, 7, S	6, 6, 7, 7, 7, 7	—	
34	Oct. 1	No. 4, 4½ mos. at 4°C.	—	7, 7, 7, 7, S, S	7, 7, S, S, S, S	—	Positive
	" 1	Human non-contact	—	5, 5, 5, 5, 5, 5	5, 5, 5, 5, 5, 7	—	

S = survived and well, 21 days.

S = survived and well, 21 days.

mice (8), kindly sent to us for comparison by Dr. Muckenfuss, proved similar to our strains. The uniformity of the seven strains was further tested by comparing the protective effect of immune monkey serum on homologous and heterologous strains.

Experiment 5.—Each of the seven strains of virus in mouse brain was injected intracerebrally into six Swiss mice. 4 days later, when the mice were prostrate,

TABLE VI

Protective Effect of Immune Monkey Serum on Homologous and Heterologous Strains of Encephalitis Virus

Virus strain No.	Source	Serum	Dose 0.03 cc. intracerebrally in dilutions	
			10 ⁻⁴	10 ⁻⁵
			Duration of life	
			days	days
3	St. Louis	Human non-contact	4, 5, 5, 5, 5, 6	5, 5, 5, 6, 6, 11
3	" "	Monkey immune	7, 7, 7, 8, 8, 8	7, 10, S, S, S, S
4	" "	Human non-contact	5, 5, 5, 6, 7, 7	4, 6, 6, 6, 7, 8
4	" "	Monkey immune	7, 7, 8, 10, 12, 12	9, 9, 10, S, S, S
5	" "	Human non-contact	5, 5, 6, 6, 7, 7	6, 6, 6, 6, 8, 9
5	" "	Monkey immune	7, 7, 7, 8, 9, S	7, 10, 11, 11, S, S
11	Kansas City	Human non-contact	5, 5, 5, 5, 5, 5	3, 5, 5, 6, 6, 6
11	" "	Monkey immune	7, 7, 7, 8, 8, 11	3, 7, 8, 9, 9, S
16	St. Louis	Human non-contact	5, 5, 5, 5, 5, 7	8, S, S, S, S, S
16	" "	Monkey immune	9, 10, S, S, S, S	S, S, S, S, S, S
22	St. Louis*	Human non-contact	5, 5, 6, 7, 7, 12	6, 7, 7, 7, 9, 11
22	" "	Monkey immune	7, 7, 8, 8, 8, 9	S, S, S, S, S, S
23	" "	Human non-contact	5, 5, 5, 5, 5, 5	6, 7, 7, 7, 8, 9
23	" "	Monkey immune	7, 7, 7, 7, 8, 9	S, S, S, S, S, S

S = survived and well, 21 days.

* Strains sent by Dr. R. S. Muckenfuss.

two from each group were killed, the brains weighed, emulsified with alundum, diluted 1 to 50 by weight with pneumococcus broth pH 7.8, and centrifuged at low speed for 10 minutes. 1 cc. of supernatant fluid was then removed and diluted further in pneumococcus broth. Final dilutions were made by combining 0.3 cc. of the virus dilution with 0.3 cc. of the normal serum or immune serum, making the concentration of virus in the serum mixtures 10⁻⁴ gm. and 10⁻⁵ gm. respectively. The normal serum was obtained from a non-contact adult in New York City; the immune serum from a *Macacus rhesus* monkey 1 week after a series of one intracerebral and three intraperitoneal injections of Strain 3 virus. The

virus-serum mixtures were incubated at 37°C. for 2 hours and then allowed to stand at 23° for 2 hours. 0.03 cc. of each dilution was then injected intracerebrally into six Swiss mice, making the actual amount of infected brain given to each mouse 3×10^{-6} gm. and 3×10^{-7} gm. respectively. The results of the titrations were expressed in duration of life in days; survivors were discarded after 21 days.

The results of this experiment are summarized in Table VI. In a series of mice receiving the greater quantity of virus, 0.03 cc. of 10^{-4} dilution, the survival time of the animals receiving immune serum was consistently longer than that of mice receiving normal serum, and in the series receiving the smaller quantity of virus, 0.03 cc. of 10^{-5} dilution, both actual protection and survival time were consistently greater in the groups receiving immune serum. Three additional tests gave similar results, indicating that immune serum prepared with Strain 3 exerted a like protective effect against Strain 3, and six other strains from St. Louis and Kansas City.

DISCUSSION

The ability of the agent we have studied to traverse membrane filters with average pore diameters as small as 66 μ differentiates it from visible microorganisms and most known viruses. Rift Valley fever virus is limited by membranes of 70 μ average pore diameter, equine encephalomyelitis is limited by membranes of about 66 μ average pore diameter (9), while only louping ill, poliomyelitis, yellow fever, and foot-and-mouth disease viruses are known to pass membranes with smaller average pore diameters. The agent is distinguishable, moreover, from the viruses of herpes, vaccine, and Japanese B encephalitis (10) in failing to provoke a response in rabbits; and it is unlike those of Rift Valley fever, louping ill, poliomyelitis, equine encephalomyelitis, and mouse encephalomyelitis (11) in its effects upon monkeys and mice. Altogether it seems clear that we are dealing with a filtrable virus differing from those previously described. It can scarcely be derived from the species of laboratory animals employed for the work, since strains coming from different laboratories and carried in different animal hosts proved alike, both in their pathogenic effects and in being neutralized by immune serum.

The specific neutralizing effect of sera from recovered cases of encephalitis in St. Louis and Kansas City, 1933 (1), will be described more fully in a subsequent paper.

CONCLUSIONS

1. The infectious agent from fatal cases of St. Louis and Kansas City encephalitis passes Seitz pads in high dilution without appreciable loss of infectivity and traverses collodion membranes with an average pore size of $66\text{ m}\mu$ or greater.

2. It is highly infectious for mice by the intracerebral and intranasal routes, but practically innocuous by the subcutaneous and intraperitoneal routes.

3. Certain strains of mice are more susceptible than others.

4. The agent administered to mice intranasally causes tremors and convulsions after a 6 to 7 day incubation period, followed by prostration and death in 8 to 10 days. Lesions are demonstrable in the olfactory bulbs 3 days after infection, in the piriform lobe after 4 days, and in Ammon's horn after 5 days.

5. In *Macacus rhesus* monkeys, the agent provokes a mild, non-fatal reaction and the development of specific neutralizing bodies. On passage in monkeys, the virus becomes progressively weaker.

6. In rabbits, guinea pigs, rats, and sheep the agent is apparently without effect.

7. All available strains of the agent proved alike in effects in animals and in immunological response.

8. The available data enable one to conclude that the agent is a filtrable virus differing from those studied heretofore.

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TRANSMISSION OF MYELOID LEUKEMIA OF MICE

ITS RELATION TO MYELOMA*

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PLATES 17 TO 20

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The experiments that will be described demonstrate that myeloid leukemia of a mammal (mouse) is transmissible and is a neoplasm that affects the precursors of granular leukocytes, the same cells producing myeloid leukemia and myeloma. Snijders (1) has shown that lymphatic leukemia of guinea pigs is transmissible, and independent studies of subsequent workers found this to be true for lymphatic leukemia of mice (2-5). The isolation of the strain of myeloid leukemia here described has made possible a study of the pathogenesis of myeloid leukemia and its relation to myeloma, and has made available a constant source of malignant myeloid cells.

Origin of Strain

Routine examination of our colony revealed a mouse (No. Ar 117) with a greatly enlarged and readily palpable spleen; some of the lymph nodes of this mouse were slightly enlarged. The leukocyte count was 105,000 per c.mm.; most of these white blood cells were myeloid cells but only 9.5 per cent of them were myelocytes with coarse basophile granules that were subsequently identified as the malignant myeloid cells characteristic of this strain (Figs. 1 to 3).

Neutrophile polynuclear leukocytes were 31 per cent, neutrophile myelocytes and metamyelocytes 20 per cent, eosinophile granulocytes 0.5 per cent, cells like monocytes 17 per cent, cells like monocytes with basophile cytoplasm (Fig. 1) 11.5 per cent, lymphocytes 10.5 per cent. The great enlargement of the spleen was due to infiltration with myelocytes containing coarse basophile granules

* This investigation has been supported by a Fund for the Study of Leukemia.

(Fig. 19). The pulp was densely packed with these cells and the lymphoid tissue atrophied. The liver was moderately enlarged and its sinusoids were distended with basophile myelocytes (Fig. 17). The femoral marrow was a dense mass of these cells (Fig. 22). The structure of the lymph nodes was retained but basophile myelocytes filled their sinuses (Fig. 20). Wherever found, many of these myeloid cells were in the process of mitotic division. Their abundant, coarse granulation, stained purple to black with Azure II, made them readily recognizable in any location.

TABLE I
Transmission of Spontaneous Myeloid Leukemia

Mice	Route of injection	No. of mice injected	No. +	Length of life of mice with myelosis
				<i>days</i>
Unrelated (R), irradiated....	i.v.	4	3	K 18, K 20, K 25
Unrelated (R), irradiated....	Intrasplenic (?)	4	4	K 27, K 31, K 48, D 50
Related (Ar), not irradiated...	i.v.	8	1	K 49
Related (Ar), not irradiated...	Intrasplenic (?)	4	2	D 66, D 109

Abbreviations Used in the Tables

+ = inoculation successful; K = killed; D = died. "Length of life" means length of life of mice with myelosis, in days, after inoculation; e.g., "K 18" means a mouse with myelosis killed 18 days after inoculation. With rare exceptions mice were killed only *in extremis*.

Route of inoculations: i.v. = intravenous; s.c. = subcutaneous; i.p. = intraperitoneal.

"Irradiated" signifies that the entire body of the animal was irradiated from 1 hour to 5 days before inoculation, with 400 r-units of X-rays, using the following factors: 190 kv., 30 ma., 50 cm. distance, 0.5 mm. Cu + 1 mm. Al. The mice were irradiated by Mr. E. Stober through the courtesy of Dr. J. R. Carty in the Department of Radiology, Cornell Medical College.

Transmission of the Leukemia

The mouse was breathing with difficulty; it was killed and an emulsion of the spleen injected into twenty mice of which eight were irradiated 1 day before inoculation. Twelve mice received injections into the tail vein, and in another group of eight mice an attempt was made to inject the material directly into the spleen, which is readily visible beneath the shaved and moistened skin. The results of these inoculations are summarized in Table I.

The material of study, the technic of inoculation and of irradiation, and the stocks of mice used have been described (5). All mice used in this study were bred in our laboratory and are members of one of three different stocks named A, R, and S. These stocks have been inbred since 1928, and were split up into a number of families that were derived from brother and sister matings. These families were designated by adding small letters to the capitals A, R, and S. Members of the different stocks (A, R, and S) are regarded as being unrelated, members of the same family (*e.g.*, Ar) as being related.

The medium used in the preparation of cell suspension was either Tyrode or Locke solution. The intravenously injected cell suspension contained from 30,000 to 100,000 leukocytes per c.mm. and was injected in amounts of 0.1 cc. Mice that appeared healthy were killed from 80 to 150 days after inoculation.

Three of the four irradiated mice inoculated intravenously were very ill about 2 weeks after inoculation; they had enlarged spleens and numerous basophile myelocytes in the blood. On postmortem examination all showed diffuse myelosis and material from these mice transmitted the disease to most of the mice inoculated with it. The similarly inoculated, unirradiated, but related mice remained healthy with the exception of one mouse that developed a nodular swelling about 1 cm. in diameter in the submaxillary region. This was thought to be an abscess and the mouse was killed 49 days after injection; but autopsy showed that it was a growth composed entirely of basophile myelocytes. Similar nodules were found in the abdominal muscles, diaphragm, and lungs, and there was a diffuse microscopic infiltration of the spleen and liver by basophile myelocytes, but the antemortem blood smear was normal. The six mice successfully inoculated intraperitoneally developed small nodules in the subcutaneous tissue at the site of needle puncture, and extensive abdominal myelomatosis. Antemortem invasion of the blood by basophile myelocytes was seen in two of these mice. The spleens and livers appeared to be normal, or showed slight diffuse or nodular infiltrations. The course of the disease was slow in the unirradiated mice and they died from 2 to 3 months after the injection.

The inference was drawn from this experiment that the disease is more readily transmitted to irradiated than to unirradiated mice. In further subpassages irradiated mice were used to propagate this strain of myeloid leukemia and unirradiated mice of the different stocks to test their susceptibility. In order to establish the optimal conditions of transfer and the anatomical changes under the various conditions of inoculations, the route of inoculation was varied, and different tissues were used for injection.

Subsequent work has shown that the disease is readily transmissible by tissues containing live basophile myelocytes; but transmission fails when they are not present. Further attempts to inoculate the spleen

directly seemed superfluous, for it was evident that these malignant myeloid cells would grow in the subcutaneous tissue or in the abdominal cavity and form tumors in these sites (Figs. 8, 9). Neither the individuality of the host nor the site of inoculation modified the characteristics of these malignant myeloid cells.

Table II is a summary of fourteen intravenous passages in irradiated mice and shows that the inoculations were successful in 88.6 per cent of the animals. The inoculations in irradiated mice by routes other than intravenous (Table III) were somewhat less successful (78 per cent). In four of the mice included among the successful inoculations in this table the growth that developed at the site of inoculation regressed. The anatomical changes in relation to the route of injection will be dealt with later.

Nodular growth about the sternum, vertebrae, and kidney, found in the mice of the first passages, suggested the attempts of producing such nodules by injecting a suspension of immature myeloid cells about these tissues. These inoculations resulted in the formation of growths at the site of injection and they were abandoned as soon as it was recognized that similar growth may be readily produced in the subcutaneous tissue and abdominal cavity.

It requires a long period for the malignant myeloid cells to form a grossly detectable growth, when introduced into the subcutaneous tissues; but similar cells produce fatal myeloid leukemia within from 2 to 3 weeks when introduced intravenously. Barely detectable nodules were occasionally found in the subcutaneous tissue 2 months after inoculation with fairly large doses, and it is probable that the incubation period can be prolonged by decreasing the inoculating dose. These data are of interest in relation to the long period of latency of some malignant neoplasms.

Inoculations are less often successful in unirradiated than in irradiated mice and the disease produced is more chronic (Table IV). Direct offspring of the mouse with the spontaneous disease (No. Ar 117) were not available for inoculation. Forty-one mice that could be traced back to the same parents as No. Ar 117 (Stock Ar) were tested and sixteen (39 per cent) proved susceptible; of forty unrelated mice (Stocks R and S) tested, six (15 per cent) proved susceptible (Table IV).

TABLE II
Intravenous Inoculations of Irradiated Mice

No. of passages	Material injected	No. of mice injected	No. +	Length of life of mice with myelosis	
				Extremes	Average
				<i>days</i>	<i>days</i>
I	Spleen	4	3	K 18 to 25	K 21
II a	"	12	10	K 17 to 21 D 20 to 48	K 20 D 27
II b	"	14	13	K 21 to 22 D 16 to 47	K 22 D 26
	Spleen (diluted)	10	8	K 83 D 27 to 48	K 83 D 36
II c	Spleen	12	11	D 20 to 24	D 22
II d	Growth	8	8	K 25 D 18 to 25	K 25 D 21
II e	"	8	8	D 28 to 54	D 37
III a	Spleen	4	4	K 21 D 21 to 24	K 21 D 22
	Blood cells	9	9	D 14 to 32	D 23
III b	Spleen	4	4	D 15 to 23	D 20
	Spleen (i.v. and s.c.)	7	7	K 29 to 41 D 21 to 25	K 33 D 24
III h	Spleen	4	0		
III f	"	8	7	K 63 D 18 to 22	K 63 D 20
III i	"	3	3	K 15, D 15	
	Spleen (diluted)	11	10	K 72 to 95 D 34 to 96	K 84 D 59
III j	Growth	9	9	D 22 to 45	D 33
III k	Growth and spleen	3	3	K 22 D 20 to 32	K 22 D 27
IV c	Spleen	2	0		
Total		132	117	(88.6 per cent)	

TABLE III

Inoculations Other than Intravenous in Irradiated Mice

No. of passages	Route of injection	Material injected	No. of mice injected	No. +	Length of life of mice with mycelosis	
					Extremes	Average
					days	days
I	i.p.	Spleen	4	4	K 27 to 48 D 50	
II a	i.p.	"	3	3	D 49 to 79	D 69
	s.c.	"	3	3	K 30 D 111 to 146	D 128
II b	s.c.	"	13	11	K 64 to 78 D 49 to 141	K 70 D 106
II c	s.c.	"	4	4	D 34 to 101	D 72
II d	Intrasplenic (?)	Growth	6	6	D 43 to 62	D 52
	Perirenal	"	5	5	D 40 to 120	D 61
	s.c.	"	6	3	K 55	
II e	s.c.	"	4	3	D 115 to 141	D 132
II f	s.c.	"	6	6	D 53 to 116	D 92
					K 70 to 98	K 84
III a	s.c.	Spleen	4	3	K 69, D 90	
III c	s.c.	Spleen*	5	2	K 87	
	Perivertebral	Spleen	5	2	D 26 to 31	D 29
	Peristernal	"	2	2	D 32, K 34	
III d	s.c.	Growth	7	6	K 84 to 125 D 43 to 95	K or D 87
III g	s.c. and i.p.	Growth and spleen	4	1	K 109	
IV b	s.c. and i.p.	" " "	4	0		
IV d	i.p.		10	10	K 70	K or D 57
					D 31 to 105	
Total.....			95	74 (78 per cent)		

* Immediately after glycerination.

Attempts at Transmission with Material Free from Viable Cells

Four different procedures that served best to demonstrate the existence of viruses capable of transmitting leukosis of chickens were used to determine whether the myeloid leukemia Strain Ar 117 can be

TABLE IV
Inoculation in Unirradiated Mice

Stock of mice	No. of passages	Route of injection	No. of mice injected	No. +	Length of life of mice with myelosis	
					Extremes	Average
					days	days
Ar	I	i.v.	8	1	K 49	
	I	Intrasplenic (?)	4	2	D 66 to 109	D 88
	II d	Perirenal	3	3	D 37 to 67	D 51
	II d	s.c.	3	2	D 129 to 145	D 137
	II f	s.c.	6	1	D 55	
	III d	s.c.	7	5	D 73 to 123, K 122	
Ara	III d	s.c.	4	1	D 48	
Arc	III d	s.c.	6	1	D 63	
Af	II e	i.v.	3	1	D 136	
	II e	s.c.	3	0		
Ak	III b	i.v.	5	0		
Rg	II e	i.v.	3	0		
	III a	i.v.	4	2	D 84 to 85	D 85
	II e	s.c.	3	1	D 135	
	III a	s.c.	4	2*	D 116	
Rf	III f	i.v.	8	0		
S	III e	i.v. and s.c.	7	0		
Total						
Stock Ar, Ara, Arc.....			41	16 (39 per cent)		
Other stocks.....			40	6 (15 per cent)		

* One mouse with transmitted myeloid tumor is still alive, 208 days after inoculation.

transmitted by material free from viable cells. These experiments, summarized in Table V, indicate that Strain Ar 117 is transmissible only by viable cells. Not a single finding was to the contrary. Some of these experiments merit further discussion.

Freezing and Thawing.—This procedure has often been used in studies undertaken to demonstrate cell-free transmission, but it has

led to erroneous conclusions, probably because of faulty technique (cf. 5). It is essential to seal in a test tube the material to be frozen, to submerge it in a liquid that is chilled to known temperature, and to

TABLE V
Inoculations with Material Free from Viable Cells

Experiments with Material Free from Viable Cells						
Material injected	Route of injection	No. of mice injected	No. +	Control		
				Material injected	No. of mice injected	No. +
I						
(a) Plasma	i.v.	4	0	Blood cells, 1/2*	5	5
(b) "	i.v.	6	0	" " 1/20	4	4
				" " 1/2	3	2
				" " 1/20	4	3
				" " 1/400	3	1
II						
(a) Dried tumor and spleen	s.c. and i.p.	5	0	Before drying	4	1
(b) Dried spleen	i.v.	5	0	" "	3	3
III						
(a) Growth frozen at -30° during 30 min.	s.c.	6	0	Not frozen	6	6
(b) Spleen frozen at -30° during 30 min.	s.c.	4	0	Frozen at -10° during 30 min.	4	4
-20° during 30 min.	s.c.	4	0	-5° during 30 min.	3	3
-15° during 30 min.	s.c.	4	0	Not frozen	3	3
(c) Growth frozen at -35° during 30 min.	i.v.	4	0	Fresh, 1/1*	3	3
				" 1/20	3	3
				" 1/400	3	3
IV						
Glycerinated spleen (7 to 21 days)	s.c.	14	0	Immediately after glycerination	5	2
Total.....		56	0	Total.....	56	46

* Dilution

* Dilutions.

keep the material at this temperature during a period of time necessary for *all* cells to reach approximately this temperature. When subjected to such treatment the malignant myeloid cells, like malignant

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mphoid cells, are destroyed at a temperature between -10° and -15°C. during 30 minutes (Table V, Experiment III b). Many cells are, however, injured at a temperature above this critical temperature, as indicated by the length of life of the inoculated animals.

Material injected	Length of life of mice with myelosis	
	Individual figures	Average
	days	days
Fresh.....	D 37, D 51, K 54	48
At -5°C. during 30 min.....	D 31, D 57, K 70	53
At -10°C. during 30 min.....	K 52, D 55, D 58, D 105	68

Since the life is lengthened by decreasing the inoculating dose, it seems probable that many cells were injured by freezing at -5° and -10°C. , and certainly all cells were destroyed at a temperature of -15°C. There seems to be a critical subzero temperature at which the cells die. Experiment IIIc shows that the unfrozen suspension caused myelosis in all mice injected, even when the dilution was 1/400, and data obtained from subsequent work (Table VI) suggest that the minimal dose producing myelosis is far below this quantity. Drying of the malignant myeloid cells *in vacuo* while in the frozen state, as described in previous reports (5), destroyed the agent concerned with the transmission of myelosis.

Plasma was obtained by spinning heparinized blood at a speed of about 1,000 R.P.M. during 5 minutes, and recentrifugalizing the plasma at a speed of about 2,000 R.P.M. during 15 minutes.

Glycerination was brought about by adding an equal volume of glycerine (c.p.) to the cell suspension. The experiment shown in Table V is of limited value because it was terminated by error 80 days after inoculation at a time when the tumors in the control mice were still small.

Relation of Dose to the Success of Inoculations; Observations on the Relation of Myeloma to Diffuse Myelosis

Qualitative experiments in search for a filterable agent, such as those shown in Table V, might depend for their success largely on the concentration of the supposed agent in the initial material. It was desirable to know the relation of the quantity of material injected to the

success of inoculation, for another reason. When a cell suspension was used for intravenous inoculation, mice that died soon after inoculation

TABLE VI
Intravenous Inoculation with Decreasing Doses

Material injected	Concentration of inoculum	No. of mice injected	No. +	Length of life	
				Extremes	Average
				days	days
(a) Blood cells	1/1	5	5	D 14 to 27	D 22
	1/20	5	5	D 20 to 32	D 25
(b) Blood cells	1/1	3	2	D 34 to 38	D 36
	1/20	4	3	D 51 to 59, K 99	D or K 70
	1/400	3	1	D 55	
(c) Spleen	1/1	3	3	D 15, K 15	D or K 15
	1/20	3	3	D 34 to 42, K 72	D or K 45
	1/400	4	4	D 35 to 77	D 51
	1/8,000	4	3	D 89 to 96, K 95	D or K 93
(d) Growth	1/1	3	3	D 22 to 35	D 27
	1/20	3	3	D 34 to 36	D 35
	1/400	3	3	D 36 to 48	D 43
(e) Growth and spleen	1/1	3	3	D 15 to 18	D 17
	1/50	3	3	D 22 to 29	D 27
	1/2,500	5	5	D 29 to 35	D 33
	1/125,000	6	6	K 41 to 46 D 48 to 65	K or D 50
(f) Growth and spleen	1/1	2	2	D 14 to 18	D 18
	1/50	4	4	D 20 to 29, K 31	D or K 26
	1/2,500	5	5	D 24 to 31 K 31 to 34	D or K 29
(g) Growth and spleen	1/1	3	3	D 17 to 21	D 19
	1/100	4	4	D 27 to 61	D or K 44
	1/10,000	5	5	D 39 to 81	D 52
	1/1 million	5	3	D 58 to 72	D 72
	1/100 million	6	0		

with a large dose showed diffuse leukemic myelosis, whereas those that lived long often died with multiple myeloma, but with no diffuse

leukemic myelosis. This observation indicated that resistance of the host as suggested by the lengthy course of the disease was the major factor in the development of multiple myelosis. Nevertheless it seemed worth while to determine if smaller doses would produce myeloma, and larger doses diffuse myelosis. The results of intravenous inoculation with decreasing doses are summarized in Table VI.

In the last experiment recorded in Table VI the concentrated cell suspension contained 110,000 leukocytes per c.mm. and the volume injected was 0.1 cc.; the mice injected with dilutions of 1/1,000,000 may therefore be estimated to have each received approximately eleven cells. In the remaining experiments the concentrated cell suspension contained from 30,000 to 100,000 leukocytes per c.mm.

Most mice used in these experiments were rendered susceptible by a preceding irradiation with X-rays. The degree of susceptibility induced by X-rays depends on the dose, and after a period of from 1 to 3 weeks following irradiation it decreases considerably (*cf.* 5). Hence if the life of the inoculated mice is lengthened by decreasing the amount of the inoculum, inoculations may become unsuccessful merely because the host is given time to regain the resistance lowered by irradiation.

It is remarkable that life is only slightly prolonged by greatly decreasing the inoculum and that the number of successful inoculations does not diminish with a delayed incubation period. The supposition that the disease would fail to develop in resistant irradiated mice unless it appeared within a few weeks because these mice had regained their resistance, previously lowered by irradiation, was contradicted by the results of the experiments. A single large dose of X-rays causes a more profound injury than hitherto suspected, or the resistance of the host is overcome by the multiplication of malignant cells during a transient period of lowered resistance. The former assumption finds support in the observations that the incidence of spontaneous lymphomatosis (6) and myelomatosis (7) is increased by a single large dose of X-rays.

The following experiment (Passage III; Experiment c of Table VI) shows the relation of the length of life to the type of the disease.

In this experiment, mice of Stock A were inoculated 3 days after irradiation with a splenic cell suspension from a mouse with myeloid leukemia. The exact number of cells injected is unknown; the concentrated cell suspension contained

120,000 white cells per c.mm., but the material had to be refiltered and an unknown number of the cells were lost during this process. Each mouse received 0.1 cc. of cell suspension. The inoculation was unsuccessful in one mouse that was killed 120 days after inoculation; all other mice died or were killed *in extremis* within 96 days after inoculation.

Length of life	Dilution of material injected	Type of disease
<i>days</i>		
K 15	1/1	Diffuse myelosis (myeloid leukemia)
K 15	1/1	" " " "
D 15	1/1	" " " "
D 34	1/20	" " " "
D 35	1/400	" " " "
D 42	1/20	" " " "
D 42	1/400	" " " "
D 50	1/400	" " " "
K 72	1/20	Multiple myeloma
D 77	1/400	Myeloma
D 89	1/8,000	"
K 95	1/8,000	Multiple myeloma
D 96	1/8,000	" "
K 120	1/8,000	Negative

All mice that died within 50 days showed diffuse myelosis; all that lived for at least 72 days showed the formation of nodules attached to bones and viscera. These masses were composed of immature myeloid cells infiltrating the surrounding tissue. Mitotic figures were numerous among the immature myeloid cells and there was no evidence of inflammation. Hence these nodules may be regarded as tumors (myelomata).

The results of all other experiments shown in Table VI were essentially the same. Tumors appeared in mice that received a small dose, whereas mice injected with a large dose developed diffuse myelosis with no tumor formation.

These experiments suggest that the type of disease is largely determined by the number of malignant myeloid cells entering the circulation. Individual resistance as a minor factor is suggested by a few irregularities in the relation of dose to the time of death. Since the resistance of all mice was very low at the beginning of the experiment, as the result of massive irradiation preceding the inoculation, and

then gradually returned, it is questionable whether a reduction of the inoculating dose did more than delay the incubation period. Not until highly susceptible unirradiated mice are available for inoculation can the effect of the dose be accurately determined.

The relation of resistance of the host to the type of disease becomes evident when mice with widely differing resistance, namely irradiated and unirradiated mice, are inoculated with the same dose.

(a) In the transmission experiment with material from the spontaneous case, irradiated and unirradiated mice were inoculated intravenously with the same amount of cell suspension. Irradiated mice: K 18, K 20, K 25, diffuse myelosis. Unirradiated mice: K 49, myeloma; K 178 (six mice), negative.

(b) Passage IIc with similar inoculation: Irradiated mice: K 29, D 29, D 53, diffuse myelosis; D 54, myeloma. Unirradiated mice: D 136, myeloma; K 147 and K 147, negative.

(c) Passage IIIa with similar inoculation: Irradiated mice: D 21, D 21, K 21, D 24, diffuse myelosis. Unirradiated mice: D 84, D 85, myeloma; K 143 and K 143, negative.

The obvious explanation of these observations is that resistant mice destroy many of the inoculated malignant myelocytes and a small number of these cells multiply and form tumors in sites other than the spleen and liver; in the latter organs they perish.

In one experiment the rôle of resistance in the pathogenesis of myelosis was tested by inoculating mice whose resistance was decreased by varying amounts of X-rays. All mice used in this experiment were approximately 3 months of age and they were members of the same family (Ar). The result of this experiment was as follows:

	No. of mice injected	No. +	No. with diffuse myelosis	No. with myeloid tumors	Length of life of mice	
					With diffuse myelosis	With myeloid tumors
					Days	Days
Unirradiated.....	8	5	1	4	D 28	D 73, K 82
Irradiated with 25 r.....	8	5	2	3	D 28 to 30	D 70, K 82
50 r.....	8	3	1	2	D 45	D 49, K 82
100 r.....	7	5	3	2	D 31 to 38	D 62, K 83
200 r.....	8	7	5	2	D 24 to 32	D 68 to 82
400 r.....	3	3	3	0	D 30 to 40	

120,000 white cells per c.mm., but the material had to be refiltered and an unknown number of the cells were lost during this process. Each mouse received 0.1 cc. of cell suspension. The inoculation was unsuccessful in one mouse that was killed 120 days after inoculation; all other mice died or were killed *in extremis* within 96 days after inoculation.

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<i>days</i>		
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K 15	1/1	" " " "
D 15	1/1	" " " "
D 34	1/20	" " " "
D 35	1/400	" " " "
D 42	1/20	" " " "
D 42	1/400	" " " "
D 50	1/400	" " " "
K 72	1/20	" " " "
D 77	1/400	Multiple myeloma
D 89	1/8,000	Myeloma
K 95	1/8,000	"
D 96	1/8,000	Multiple myeloma
K 120	1/8,000	" "
		Negative

All mice that died within 50 days showed diffuse myelosis; all that lived for at least 72 days showed the formation of nodules attached to bones and viscera. These masses were composed of immature myeloid cells infiltrating the surrounding tissue. Mitotic figures were numerous among the immature myeloid cells and there was no evidence of inflammation. Hence these nodules may be regarded as tumors (myelomata).

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(b) Passage IIc with similar inoculation: Irradiated mice: K 29, D 29, D 53, diffuse myelosis; D 54, myeloma. Unirradiated mice: D 136, myeloma; K 147 and K 147, negative.

(c) Passage IIIa with similar inoculation: Irradiated mice: D 21, D 21, K 21, D 24, diffuse myelosis. Unirradiated mice: D 84, D 85, myeloma; K 143 and K 143, negative.

The obvious explanation of these observations is that resistant mice destroy many of the inoculated malignant myelocytes and a small number of these cells multiply and form tumors in sites other than the spleen and liver; in the latter organs they perish.

In one experiment the rôle of resistance in the pathogenesis of myelosis was tested by inoculating mice whose resistance was decreased by varying amounts of X-rays. All mice used in this experiment were approximately 3 months of age and they were members of the same family (Ar). The result of this experiment was as follows:

	No. of mice injected	No. +	No. with diffuse myelosis	No. with myeloid tumors	Length of life of mice	
					With diffuse myelosis	With myeloid tumors
					days	days
Unirradiated.....	8	5	1	4	D 28	D 73, K 82
Irradiated with 25 r.....	8	5	2	3	D 28 to 30	D 70, K 82
50 r.....	8	3	1	2	D 45	D 48, K 82
100 r.....	7	5	3	2	D 31 to 35	D 52, K 83
200 r.....	8	7	5	2	D 24 to 32	D 68 to 82
400 r.....	3	3	3	0	D 30 to 40	

The increase in the number of mice with diffuse myelosis and in the number of successful inoculations suggests that the smallest dose of X-rays that increases susceptibility to transmissible myelosis is approximately 100 r (*cf.* 5).

Blood Changes

Since the diagnosis of myelosis of Strain Ar 117 can be made by recognition of one or two malignant cells in the blood smear it seemed superfluous to undertake an elaborate quantitative study of morphological changes in the circulating blood. Two procedures were used to obtain malignant cells as free from other cells as possible: (*a*) inoculation of mice whose blood-forming tissues were depleted by irradiation; (*b*) production of tumor by subcutaneous or intraperitoneal inoculation of the neoplastic blood cells. These procedures showed that the malignant cells of Strain Ar 117 are myelocytes with coarse basophile granules that differ morphologically from non-malignant immature myeloid cells (Figs. 2, 3, 11 to 16).

The size of these cells in fixed smears stained with Wright and Giemsa solutions varies greatly; their diameter is from 6 to 14 μ , with an average of about 10 μ . The nucleus is vesicular or slightly indented or lobed. The cytoplasm is slightly or moderately basophilic and contains coarse granules that fail to stain with eosin and hematoxylin, but stain readily with Azure II. In blood smears there are often seen detached cytoplasmic masses with basophilic granules, as shown in Figs. 1 and 2. The color of these granules varies somewhat in preparations stained with Romanowsky dyes; it is usually dark purple and sometimes it is reddish or very dark and almost black. They do not give the oxydase reaction with Schultze's indophenyl blue.

It is doubtful whether the mouse possesses basophile granular leukocytes (mast cells), such as are found in man (8), but the common promyelocytes of the mouse have basophile granules somewhat smaller than those of the basophile myelocyte of Strain Ar 117 (Fig. 5). Tissue mast cells, readily demonstrable in lymph nodes of the mouse stained with eosin-azure according to Maximow, and occasionally seen in smears of lymph node, spleen, or heart blood post mortem, are much larger than myelocytes, their granules are much more abundant and more darkly stained (Fig. 6). Transitional forms between the tissue mast cells and the granular leukocytes of the bone marrow were never seen.

In view of these considerations the characteristic cell of Strain Ar 117 may be regarded as a pathological (malignant) myelocyte.

The number of these cells in the blood stream of the mouse with the spontaneous disease was small (9.5 per cent). The blood smears of irradiated mice that were inoculated intravenously showed, however, numerous basophile myelocytes and almost no other cells. In mice inoculated subcutaneously or intraperitoneally the blood appeared normal during a period of several months, but often there was terminal leukemia. The blood smears of mice with multiple myeloma were, in most instances, free from basophile myelocytes. Table VII shows some figures that illustrate these observations. Myelosis caused by Strain Ar 117 is associated with severe anemia.

Fig. 4 shows the blood smear of a mouse with spontaneous myeloid leukemia (No. Af 372). Transmission of this instance of myeloid leukemia was attempted by inoculation of 79 mice, including 43 irradiated mice and eleven unirradiated closely related mice, but without success.

Anatomical Changes; Observation on the Pathogenesis

The experiments described indicate that the myeloid cells in the new host originate from those that are introduced and are not derived from those of the inoculated animal. (a) Transmission is only successful with viable cells. (b) It is more successful in mice whose blood-forming organs have been injured than in mice with uninjured blood-forming organs. (c) Tumors composed of cells indistinguishable from those injected are formed at the site of injection. The anatomical changes, however, differ somewhat, according to the route of introduction.

In all instances in which subcutaneous inoculation was successful there was a tumor at the site of injection (Figs. 8, 21).

These tumors were just palpable from 6 to 12 weeks after inoculation and grew slowly until they reached from 3 to 5 cm. in diameter. The affected mice died from 3 to 5 months after the injection. Large subcutaneous tumors often ulcerated and in a small number of mice they regressed. From the subcutaneous tissue the tumors occasionally invaded by continuity the peritoneal and pleural cavities. Here they formed diffuse infiltrations or tumor nodules upon the peritoneal and pleural surfaces and were associated with milky effusions in large quantity. Smear preparations of this effusion (Fig. 5) showed basophile myelocytes and a few very large mononuclear cells which were probably degenerated mesothelial cells, but

TABLE VII
Blood Counts of Mice with Transmitted Myelosis Strain Ar 117

No. of mouse	Route of inoculation	Date of				White cell count per cmm.	Red cell count per c.mm.	Differential count				
		Inoculation	Irradiation	Examination	Death			Baso-philic myelo-cytes	Poly-nucleus	Lym-pho-cytes	Monoc-ytes	Un-typed
R 3187	i.v.	1934 Apr. 5	1934 Apr. 4	1934 Apr. 25	1934 K Apr. 25	25,000	1,075,000	per cent 25	per cent 41	per cent 10	per cent 17	per cent 7
A 8565	i.v.	" 5	" 5	May 9	K May 9	54,000	1,580,000	83	45	6.5	4	2
A 9001	i.v.	July 31	July 31	Sept. 13	K Sept. 13	83,000	830,000	46	41	5.5	4.5	3
A 8610	s.c.	Apr. 12	—	May 23 June 14 Aug. 29	— — K Sept. 4	Normal " Moderate in-crease	— — —	0 0 0	47 50 77	44 47 5	4 0 16	5 3 2
A 8573	s.c.	Apr. 5	Apr. 5	June 7 " 14 " 21 " 22	—	Slight increase " " "	— — — —	0 0 0 4	42 46.5 76 42	42 29.5 15 33	12.5 20.5 7.5 17	3.5 3.5 1.5 4
R 3201	(Control to R 3187)	—	Apr. 5	Apr. 5	—	8,000	10,150,000	0	77	16.5	4	2.5
Normal mice (4)		—	—	—	—	—	—	0	45.6	53.4	1	—

Notes on Table VII

Certain difficulties were encountered in making differential counts. (a) Some of the basophile myelocytes were stripped of their granules in the preparation of the smears and hence could not be identified. (b) In fixed blood smears of mice differential diagnosis of monocytes from myelocytes was often difficult because the neutrophilic granules were in many instances slightly stained or unstained, in our preparations. (c) Some small cells with hyperchromatic nuclei and scarcely any cytoplasm, classified by us among the lymphocytes, were possibly micromyelocytes or myeloblasts. It is probable that these difficulties could be overcome by study of vitally stained blood smears. Immature normal myeloid cells were counted with the polynuclear leukocytes.

In irradiated Mice R 3187, A 8565, and A 9001 myeloid leukemia was produced by intravenous inoculation. No. R 3201 is a control to No. R 3187. In the three leukemic mice there was profound anemia. Decrease of the number of lymphocytes was largely due to irradiation. No. A 8610, an unirradiated, and No. A 8573, an irradiated mouse, were successfully injected by the subcutaneous route. Blood smears made repeatedly from the first mouse during its chronic illness gave no indication of leukemia. In the second mouse a few myelocytes appeared in the blood in the terminal stage of the disease. All of these mice were killed when they appeared to be very ill.

no microorganisms. Distant foci of metastases were found in mice that died or were killed at an advanced stage of the disease. The smears from the blood of these mice appeared normal or more often in the terminal stage of the illness showed basophile myelocytes. Metastatic infiltrations consisted of multiple white, firm tumors similar to the primary tumors and they were found attached to the bones, for example the rib, mandible, vertebra, bones of the skull and of the extremities, or in muscles and viscera. There was often mild diffuse myelosis of spleen and liver.

After intraperitoneal inoculation there was extensive distention of the abdomen caused by the formation of numerous partly confluent tumors attached to the mesentery, diaphragm, and peritoneum (Fig. 9). In the peritoneal cavity there was much milky effusion in which the cells were largely basophile myelocytes (Fig. 3). Blood invasion and formation of metastatic foci were similar to those observed in mice injected subcutaneously.

After intravenous inoculation the changes were different in mice that died from 2 to 5 weeks after injection, from those in mice that died at a later date, but the time relation is only approximate. The former developed diffuse leukemic myelosis (Fig. 7); the latter developed myelomata with or without the diffuse systemic disease (Fig. 10).

In order to study the early manifestations of the disease, irradiated mice inoculated intravenously were killed 4, 8, 12, and 18 days after inoculation.

As early as 4 days after the inoculation, basophile myelocytes were numerous in the pulp of the spleen. Their number was considerable 8 days after injection and they began to accumulate in the sinusoids of the liver and in the bone marrow. Their number per unit area was greatest in the spleen (Fig. 19), almost as great in the bone marrow (Fig. 22), and much less in the liver (Fig. 18). They multiplied in these sites by mitosis. As they increased in the pulp of the spleen the lymphoid follicles became smaller. In some mice mitosis was abundant in the lymphoid tissues, apparently as the result of regeneration following injury caused by X-rays. The malignant myeloid cells showed progressive growth in the sinusoids of the spleen. These became distended with basophile myelocytes and contained only a few erythrocytes. In the lymph nodes there was slight accumulation of malignant myeloid cells in the lymph sinuses (Fig. 20). In several mice with diffuse myelosis there was a diffuse infiltration about the thoracic vertebrae and costovertebral angle, involving the muscle tissue and often causing weakness of hind legs.

DISCUSSION

The experiments described here bring experimental proof for the assumption that myeloid leukemia of a mammal (mouse) is a neoplasm. The malignant cells of the strain described are basophile myelocytes and they produce the systemic disease with leukemia but with no tumor formation in some animals, and multiple myeloma alone in others.

The strain described has well defined characteristics that have remained constant throughout this study. These are: morphological peculiarities of the malignant myelocytes, relatively chronic course of illness, ability to form tumors in the injected subcutaneous tissue with little tendency to invade the circulating blood. It is not probable that all myeloid leukemias of mice that will prove transmissible will behave in the same way. Experience with five strains of lymphoid leukemia (5) points to the contrary, since careful analysis of these transmissible strains has revealed distinguishing characters in each. More recent experience with lymphoid leukemia of mice has confirmed these observations. Several strains have been observed that did not possess the ability to form tumors in the subcutaneous tissue; one strain was associated with icterus and erythroblastosis.

An explanation of the difficulty of transmitting myeloid neoplasms is desirable since procedures such as those successfully applied to spontaneous myelosis Ar 117 have failed in numerous instances of similar disease.

Two factors are significant in determining whether diffuse myelosis or myeloma is produced; namely, the route of entry and the resistance of the host. A third factor, the inoculating dose, has been discussed in the text. Spontaneous multiple myeloma may be either multicentric in origin, such as the bone sarcoma produced by irradiation (*cf.* Martland (9)) or may be unicentric and develop after intravenous dissemination as in our resistant mice. Studies of lymphomatosis (*cf.* 5) indicate that there is a fourth factor that is probably highly significant in determining the type of myelosis; namely, the character of the malignant myeloid cells. This factor can be revealed only by transmission experiments made with several strains of myelosis, under identical conditions.

The experimental disease produced by the malignant cells may be acute and destroy the mouse within from 2 to 3 weeks, or it may be chronic, causing the first manifestations of illness after about 2 months, progressing slowly, and causing the death of the animal from 4 to 5 months after inoculation. Variations in the route of entry, resistance of the host, and inoculating dose may account for these differences. Thus the classification of leukemias into acute and chronic forms is highly arbitrary and does not distinguish different types of diseases (*cf.* 5).

A single factor, *e.g.* resistance of the host, may delay the course of illness and modify its type. It has been shown that after intravenous inoculation with a large dose, irradiated mice (animals with low resistance) die within 2 to 3 weeks with systemic leukemic myelosis, whereas unirradiated mice of the same moderately resistant stock develop, instead of systemic myelosis, multiple myeloma. Evidently the resistant mice employed had the ability to destroy the malignant cells in the spleen and liver, but they were unable to cope with the malignant cells that lodged in some tissues favorable for their growth where they multiplied and produced tumors. The soil favorable to the best growth may differ with each malignant cell and may determine some of the characteristic features of the strain. For example, the malignant myelocytes of Strain Ar 117 grow well in areolar and muscle tissue; the malignant lymphocytes of our Strain Rg 10 favor the ovaries, and bilateral ovarian lymphosarcoma may be the only gross evidence of successful inoculation with this strain (*cf.* 5).

Leukemias and tumors composed of either myeloid or lymphoid blood cells are similar malignant diseases, the manifestations of which depend on numerous factors, for example (*a*) character of the malignant cell, (*b*) route of entry (point of origin), (*c*) resistance of the host, (*d*) inoculating dose (*i.e.* number of cells that have undergone malignant transformation). The observation that with greatly decreased doses the course of the disease is only slightly lengthened is significant in relation to the effect of X-rays in leukemia. Irradiation of the entire body of leukemic mice with X-rays and radium prolongs the life of the animals, probably because these rays destroy many malignant blood cells (10).

SUMMARY

A transmissible strain of myeloid leukemia of mice is described. It can be readily passed from diseased to healthy mice by the transfer of tissues that contain live cells; but inoculation fails when the latter are not present.

Inoculation is successful in almost every mouse whose resistance is lowered by X-rays. It is often successful in mice related to the animal in which the spontaneous leukemia took its origin, and occasionally successful in mice unrelated to it.

The systemic diffuse disease (myeloid leukemia) is produced only by intravenous inoculation with relatively large doses, whereas subcutaneous or intraperitoneal inoculation results in the formation of tumors composed of myelocytes with basophile granules, in other words malignant blood cells of the strain described.

Intravenous inoculation with small doses in susceptible mice, or similar inoculation with larger doses in somewhat resistant mice, results in the formation of tumors composed of myelocytes (multiple myeloma).

CONCLUSIONS

Experiments demonstrate that transmissible myeloid leukemia of mice is a neoplasm. The malignant immature granulocytes that produce the systemic disease may also produce tumors (multiple myeloma).

Some factors that determine the type of transmitted myelosis are (a) the resistance of the host, (b) the route of entry, (c) the character of the malignant cell, and, probably, (d) the inoculating dose.

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EXPLANATION OF PLATES

All blood smears were stained with Wright and Giemsa solutions, and the sections with eosin and Azure II solutions. The magnifications stated are approximate.

PLATE 17

FIG. 1. Blood smear from spontaneous myeloid leukemia of Mouse Ar 117, showing two myelocytes with basophile granules, one immature polymorphonuclear leukocyte, one mature polymorphonuclear leukocyte, one unidentified large mononuclear cell, a small lymphocyte, and a cytoplasmic mass with basophile granules. $\times 910$.

FIG. 2. Transmitted myeloid leukemia with myelocytes, an unidentified mononuclear cell, and a cytoplasmic mass with basophilic granules in the field. $\times 910$.

FIG. 3. Smear of peritoneal effusion that accompanied abdominal myelomatosis produced by transmission. $\times 910$.

FIG. 4. Blood smear in spontaneous myeloid leukemia of Mouse Af 372; (a) myeloblast; (b) and (c) myelocytes; (d) immature polymorphonuclear leukocyte; (e) mature polymorphonuclear leukocytes; (f) cytoplasmic mass containing neutrophile granules. $\times 910$.

FIG. 5. A normal myelocyte from a smear of the spleen of a mouse with non-malignant myeloid metaplasia. $\times 910$.

FIG. 6. Tissue mast cell from the lymph node of a healthy mouse. $\times 910$.

PLATE 18

FIG. 7. Diffuse myelosis (myeloid leukemia) produced by transfer of myeloid cells to an irradiated mouse. The spleen is greatly enlarged, the liver is moderately enlarged, and there are numerous small hemorrhagic infarcts in the lung.

FIG. 8. Myeloid tumor produced by subcutaneous inoculation. The tumor occupies most of the right lateral aspect of the mouse. There is no evidence of metastases.

FIG. 9. Abdominal myelomatosis produced by intraperitoneal injection. The abdominal cavity is filled with tumor masses, diffusely infiltrating the mesentery and omentum.

FIG. 10. Multiple myelomata in the left submaxillary region, ribs, vertebrae, muscles of the abdominal wall, and extremities, produced by intravenous inoculation of a moderately resistant, unirradiated mouse.

PLATE 19

FIG. 11. Myeloid leukemia produced by transmission to an irradiated mouse. There are nine myelocytes with basophile granules in the field. $\times 400$.

FIGS. 12 to 16. Myelocytes with basophile granules and one neutrophile polymorphonuclear leukocyte (Fig. 13). Magnifications Figs. 12, 13, 15, 16 $\times 850$; Fig. 14 $\times 400$.

FIG. 17. The liver of a mouse (No. Ar 117) with spontaneous myeloid leukemia. $\times 200$.

FIG. 18. Higher magnification of the liver of a mouse with transmitted myeloid leukemia showing distention of the sinusoids by immature myeloid cells with compression of the liver cells. $\times 750$.

PLATE 20

FIG. 19. Extensive infiltration of the spleen with compression of a lymphoid follicle in a mouse with myeloid leukemia produced by transmission. $\times 180$.

FIG. 20. Accumulation of immature myeloid cells in the sinuses of a lymph node in a mouse (No. Ar 117) with spontaneous myeloid leukemia. $\times 200$.

FIG. 21. Immature myeloid cells infiltrating the abdominal muscle after subcutaneous inoculation. $\times 150$.

FIG. 22. Extensive infiltration of the bone marrow with myelocytes containing coarse basophile granules in myeloid leukemia caused by transmission. $\times 600$.

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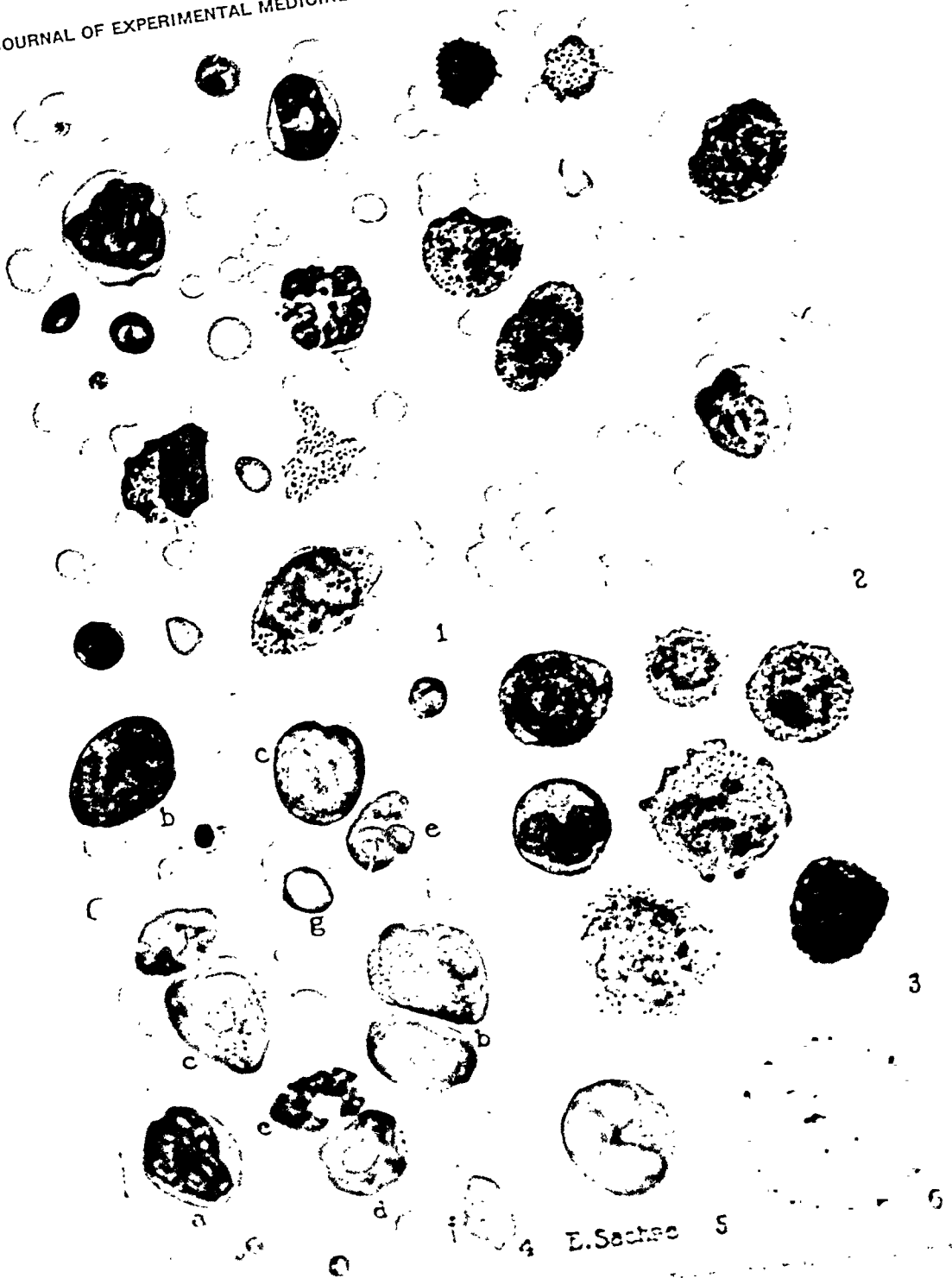
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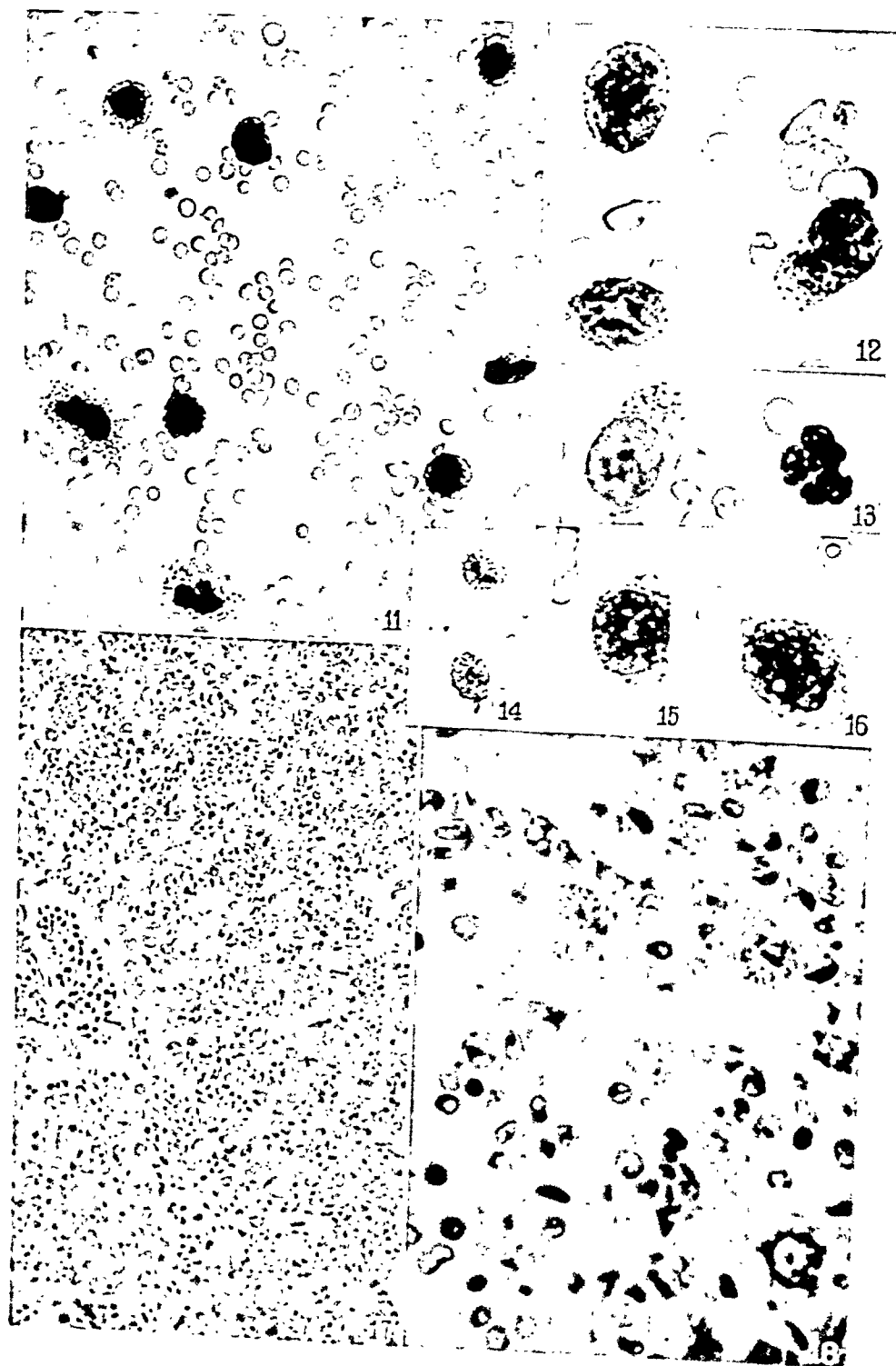
FIG. 21. Immature myeloid cells infiltrating the abdominal muscle after subcutaneous inoculation. $\times 150$.

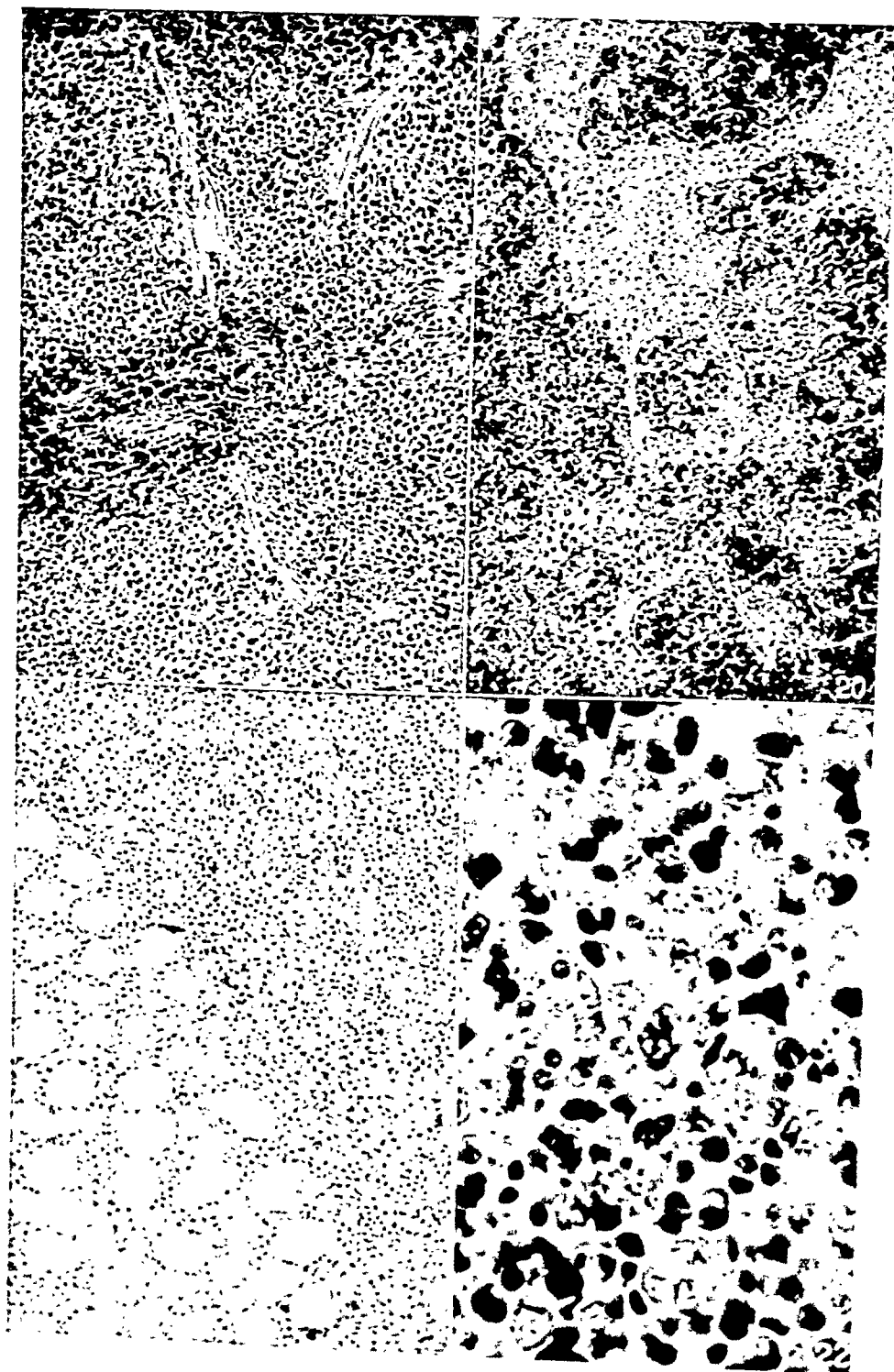
FIG. 22. Extensive infiltration of the bone marrow with myelocytes containing coarse basophile granules in myeloid leukemia caused by transmission. $\times 600$.











THE NEUTRALIZATION TEST IN POLIOMYELITIS

COMPARATIVE RESULTS WITH FOUR STRAINS OF THE VIRUS*

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During the last two decades various methods of determining the power of human sera to neutralize poliomyelitis virus have been employed, and from them various clinical and epidemiologic conclusions have been drawn. Few will deny that these studies have added to knowledge with regard to poliomyelitis but at the same time there is a growing belief that from some of them, perhaps, attempts have been made to obtain more clinical and epidemiologic information than the methods afford. For instance, although it is open to doubt whether the methods used to make these determinations in clinical poliomyelitis are very accurate, one fact is becoming increasingly apparent; namely, that in neutralization tests with poliomyelitis virus, different results may be obtained with some human sera when tested with different strains of virus. Thus certain convalescent sera tested with a strain which has been recently isolated from a human case will act differently than when tested with a strain which has been subjected to a long series of monkey passages. It is our present purpose to report similar comparative tests on sera obtained from normal human adults who did not give a history of having sustained a recognizable attack of poliomyelitis, and to discuss them in the light of previous results with sera from convalescent and normal persons representing younger age groups. The purpose of such a discussion mainly concerns the specificity of the antigen used in such neutralization tests on human sera. In experimental work in this disease anti-

*The expenses of this investigation have been defrayed by a grant from the National Research Council.

paring the two groups, Shaughnessy, Harmon, and Gordon (8) found that antiviral titres of individual samples of normal sera appeared to be more potent in their action against the virus than were those of the sera of convalescents. Brodie (9) found that in 70 per cent of his normal adult sera the neutralizing power was about four-fifths that of pooled convalescent sera, and in 30 per cent it was equal in titre. Subsequently Howitt (10) pointed out that the average power of individual samples of convalescent sera to neutralize poliomyelitis virus was perhaps less than had been generally supposed. This fact becomes even more evident if one compiles from the literature the available series of tests on individual samples of serum in which virus: serum mixtures have been set up in a dilution of 1:1, 1:2, or 1:3, and compares the normal with the convalescent results according to age groups. In fact, with the exception of Aycock's convalescent findings (7) (which are not included because they were based on pooled lots of sera), the average neutralizing power of individual samples of convalescent sera reported in the literature seems to be uniformly less in all age groups than that of normal sera. The many observations on this point will not be transcribed in detail but general trends of the results by different investigators are shown by the graphs in Fig. 1. In this figure we have included only those series of tests which have been reported since 1929. Prior to that date the number of reported tests in different age groups was small and certain variations in technique render a comparison with the recent work somewhat difficult. There are still technical differences which involve some of the results shown in Fig. 1, but only those tests have been included in which virus: undiluted serum mixtures have been set up in a proportion of 1:1, 1:2, or 1:3.

With the results expressed as they are in Fig. 1, and in so far as the data go, it will be seen that individuals from rural districts have less than the normal, average amount of antiviral substance, and that contacts appear to have a little above the average, as do also individuals from regions where poliomyelitis is thought to be rare. There is no evidence that the acquisition of the clinical disease in the remote or recent past, gives rise to an increase in antiviral substance when tested with passage strains, although it is unfortunate that so few tests are reported in the literature which have been done on convalescent sera from children.

We shall review next the question of variation in the virus and the comparisons between neutralization tests performed with different strains. That old passage strains of poliomyelitis virus may exhibit slight immunological differences, one from another, was described in 1929 by Stewart and Rhoads (21). That more easily demonstrable differences existed between a strain recently isolated from a human case, and a strain which had been passed through monkeys many times was observed in 1931 by Burnet and Macnamara (22). The principles of their observation have subsequently been confirmed (23, 24, 20, 25). There is no evidence to show that all such recently isolated strains are alike, and the actual number of series of neutralization tests on human sera in which results with several strains have been compared is few. Nevertheless, evidence has appeared in the literature

viral properties appear in the blood of the convalescent or immunized monkey, and, more or less coincidentally, the animal may be found to be immune to a subsequent intracerebral inoculation of the virus. In human sera (both convalescent and normal) the presence of substance which neutralizes poliomyelitis virus has, by analogy therefore, been taken as a measure of "immunity," although it is recognized, of course, that the extent to which the mere presence of such neutralizing antibodies represents immunity is a matter of some speculation. But the difference between the situations in the monkey and in man is that in the monkey neutralization tests have usually been made with the actual strain which induced the experimental disease, whereas most human sera have been tested with strains of virus which may be quite different from those which induce the human disease. As a result it now seems questionable whether human immunity to poliomyelitis has been very accurately determined by the methods employed in the past and it will be with this fact that this paper will be concerned.

The early methods of performing virus neutralization tests in poliomyelitis, the results, and their interpretations will not be reviewed because the reader may be referred to a recent article by Wells (1) for a summary of these subjects. In the majority of these and subsequent studies it was assumed that the presence in human blood of a given amount of substance which neutralized poliomyelitis virus was a reliable test for immunity in this disease. On this basis comparisons have been made between tests performed on sera from patients who had sustained frank attacks of poliomyelitis, from contacts, and from individuals who were not known to have been contacts or to have sustained a recognized attack of the disease. Interpretations have also been drawn from comparisons made between tests on the sera of normal individuals from this country as opposed to those from regions where clinical poliomyelitis is thought to be rare, such as: Porto Rico (2), Liberia (3), Greenland (4), and certain parts of China (4, 5). Similar interpretations have been drawn from the results of tests on normal individuals from the northern as opposed to the southern part of this country (6); and on normal children and adults from rural districts as opposed to normal urban individuals of the same age groups (7, 8). All of these tests were performed with strains of the virus which had been passed through monkeys many times.

Almost from the beginning of this work it became apparent that the average neutralizing power of sera from normal, adult individuals did not differ much from that of the sera of convalescents, most of those tested also being adults. In com-

paring the two groups, Shaughnessy, Harmon, and Gordon (8) found that antiviral titres of individual samples of normal sera appeared to be more potent in their action against the virus than were those of the sera of convalescents. Brodie (9) found that in 70 per cent of his normal adult sera the neutralizing power was about four-fifths that of pooled convalescent sera, and in 30 per cent it was equal in titre. Subsequently Howitt (10) pointed out that the average power of individual samples of convalescent sera to neutralize poliomyelitis virus was perhaps less than had been generally supposed. This fact becomes even more evident if one compiles from the literature the available series of tests on individual samples of serum in which virus: serum mixtures have been set up in a dilution of 1:1, 1:2, or 1:3, and compares the normal with the convalescent results according to age groups. In fact, with the exception of Aycock's convalescent findings (7) (which are not included because they were based on pooled lots of sera), the average neutralizing power of individual samples of convalescent sera reported in the literature seems to be uniformly less in all age groups than that of normal sera. The many observations on this point will not be transcribed in detail but general trends of the results by different investigators are shown by the graphs in Fig. 1. In this figure we have included only those series of tests which have been reported since 1929. Prior to that date the number of reported tests in different age groups was small and certain variations in technique render a comparison with the recent work somewhat difficult. There are still technical differences which involve some of the results shown in Fig. 1, but only those tests have been included in which virus: undiluted serum mixtures have been set up in a proportion of 1:1, 1:2, or 1:3.

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to suggest that the situation shown in Fig. 1 would be altered if the tests had been done with so called "human" strains.¹

For instance, in comparing the neutralizing action of different human sera on their Australian human strain and on a New York passage (M V) strain, Burnet and Macnamara (22) found that although pooled, convalescent sera would neutralize both strains, a few tests with individual samples of convalescent sera failed to show this parallelism, in that only the human strain was neutralized.

Later Howitt (20) made similar, comparative observations upon seven samples of adult, convalescent sera. All of these convalescent sera neutralized her 1931 human strain, whereas less than half of these sera neutralized her passage (M V) strain. Howitt concluded that in judging the neutralizing ability of human sera it would seem preferable to employ a virus that had not undergone too great a modification by repeated transfer through monkeys. It is worth noting that the conclusion was based on work with a human strain which was not derived from the epidemic in which Howitt's patients had sustained their attacks of poliomyelitis.

We (25) have also performed this type of experiment upon sera obtained from children in which tests with our human (W—7th passage) strain were compared to those of a passage (M) strain. The children tested were either normal, contacts, or in early or late convalescent stages of an abortive or frank attack of poliomyelitis. Their exposure to, or acquisition of the disease occurred in the same epidemic from which the human strain was isolated. From the results, and in spite of the present difficulties inherent in the technique, it has seemed that those tests performed with the human strain gave a better concept of antiviral properties induced by the human disease than did similar tests performed with the passage strain.

It has been our object in the present study to amplify the previously reported results by performing a comparative series of tests on samples of sera from normal adults using two human and two passage

¹ Hereafter the term human strain will be used in this article to designate a strain recently isolated from a case of human poliomyelitis. The terms human and passage in referring to strains of poliomyelitis virus have not been defined. They are employed in this article because of priority of usage (23, 24). Criticism might be raised against our use of the term human, in referring later to seventh and tenth passage strains. As it is almost impossible at present to perform neutralization tests with strains in their first few passages because of their low virulence for the monkey, the term human strain will be defined in this article as referring to a strain which has not been passed many times beyond the point at which it is suitable for use in neutralization tests. Passage strains represent those strains passed many times beyond this point. Although both our human strains (W and F) came from the same epidemic, the W strain should perhaps be qualified by the terms,—1931, New Haven (seventh passage); and the F strain as,—1931, New York (tenth passage).

strains of the virus. The experiments have also been devised to throw some light on the following questions: (a) To what extent do human strains act like one another when tested by this method? (b) To what extent do passage strains act like one another in this respect? (c) What is the power of normal adult sera to neutralize human poliomyelitis virus? (d) To what extent do the findings in these human strain experiments alter certain concepts with regard to clinical aspects, and to the epidemiology of poliomyelitis based on passage strain neutralization experiments?

Methods

Strains Employed.—Four strains of poliomyelitis virus were used. They included two human strains, designated as W and F; and two passage strains, designated as Aycock and M.

Human Strains

W Strain, New Haven, 1931.—Our W strain was in its seventh passage. Obtained in 1931 from the throat of a child in the 1st day of an abortive attack of poliomyelitis, its isolation (26) and its first seven monkey passages (25) have been described. Material employed for all neutralization tests came from a single animal (Monkey B-6); a procedure which was followed in previous neutralization tests described with this strain.² The supernatant fluid (after centrifugalization) from a 10 per cent suspension of ground cord, when mixed with an equal part of normal monkey serum and injected intracerebrally in 0.5 cc. amounts, has infected nine fresh monkeys thus tested. The experimental disease so produced is clear-cut but usually not fatal. In nine monkeys so infected, the incubation period to the onset of fever averaged about 6 days; the febrile period prior to the onset of paralysis about 4 days; and the mortality in this series of monkeys was about 12 per cent.

F Strain, New York, 1931.—This was obtained through the kindness of Dr. Simon Flexner of The Rockefeller Institute for Medical Research. It had been isolated during the summer of 1931 from the medulla and spinal cord of a fatal case of poliomyelitis and the sample we received represented the eighth passage. A virulence titration at this time showed that 0.5 cc. of a 1 per cent suspension of this material would infect a monkey but the experimental disease so produced was mild, whereas when a similar amount of a 5 or 10 per cent suspension was injected it was severe.

Material from a single animal (No. 1-97) representing the tenth passage was

² Monkey B-6 had contracted a severe form of the experimental disease. For passing human strains it is our practice to select those animals which are most extensively paralyzed.

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We (25) have also performed this type of experiment upon sera obtained from children in which tests with our human (W—7th passage) strain were compared to those of a passage (M) strain. The children tested were either normal, contacts, or in early or late convalescent stages of an abortive or frank attack of poliomyelitis. Their exposure to, or acquisition of the disease occurred in the same epidemic from which the human strain was isolated. From the results, and in spite of the present difficulties inherent in the technique, it has seemed that those tests performed with the human strain gave a better concept of antiviral properties induced by the human disease than did similar tests performed with the passage strain.

It has been our object in the present study to amplify the previously reported results by performing a comparative series of tests on samples of sera from normal adults using two human and two passage

¹ Hereafter the term human strain will be used in this article to designate a strain recently isolated from a case of human poliomyelitis. The terms human and passage in referring to strains of poliomyelitis virus have not been defined. They are employed in this article because of priority of usage (23, 24). Criticism might be raised against our use of the term human, in referring later to seventh and tenth passage strains. As it is almost impossible at present to perform neutralization tests with strains in their first few passages because of their low virulence for the monkey, the term human strain will be defined in this article as referring to a strain which has not been passed many times beyond the point at which it is suitable for use in neutralization tests. Passage strains represent those strains passed many times beyond this point. Although both our human strains (W and F) came from the same epidemic, the W strain should perhaps be qualified by the terms,—1931, New Haven (seventh passage); and the F strain as,—1931, New York (tenth passage).

strains of the virus. The experiments have also been devised to throw some light on the following questions: (a) To what extent do human strains act like one another when tested by this method? (b) To what extent do passage strains act like one another in this respect? (c) What is the power of normal adult sera to neutralize human poliomyelitis virus? (d) To what extent do the findings in these human strain experiments alter certain concepts with regard to clinical aspects, and to the epidemiology of poliomyelitis based on passage strain neutralization experiments?

Methods

Strains Employed.—Four strains of poliomyelitis virus were used. They included two human strains, designated as W and F; and two passage strains, designated as Aycock and M.

Human Strains

W Strain, New Haven, 1931.—Our W strain was in its seventh passage. Obtained in 1931 from the throat of a child in the 1st day of an abortive attack of poliomyelitis, its isolation (26) and its first seven monkey passages (25) have been described. Material employed for all neutralization tests came from a single animal (Monkey B-6); a procedure which was followed in previous neutralization tests described with this strain.² The supernatant fluid (after centrifugalization) from a 10 per cent suspension of ground cord, when mixed with an equal part of normal monkey serum and injected intracerebrally in 0.5 cc. amounts, has infected nine fresh monkeys thus tested. The experimental disease so produced is clear-cut but usually not fatal. In nine monkeys so infected, the incubation period to the onset of fever averaged about 6 days; the febrile period prior to the onset of paralysis about 4 days; and the mortality in this series of monkeys was about 12 per cent.

F Strain, New York, 1931.—This was obtained through the kindness of Dr. Simon Flexner of The Rockefeller Institute for Medical Research. It had been isolated during the summer of 1931 from the medulla and spinal cord of a fatal case of poliomyelitis and the sample we received represented the eighth passage. A virulence titration at this time showed that 0.5 cc. of a 1 per cent suspension of this material would infect a monkey but the experimental disease so produced was mild, whereas when a similar amount of a 5 or 10 per cent suspension was injected it was severe.

Material from a single animal (No. 1-97) representing the tenth passage was

² Monkey B 6 had contracted a severe form of the experimental disease. For previous human strains it is our practice to select those animals which are most extensively paralyzed.

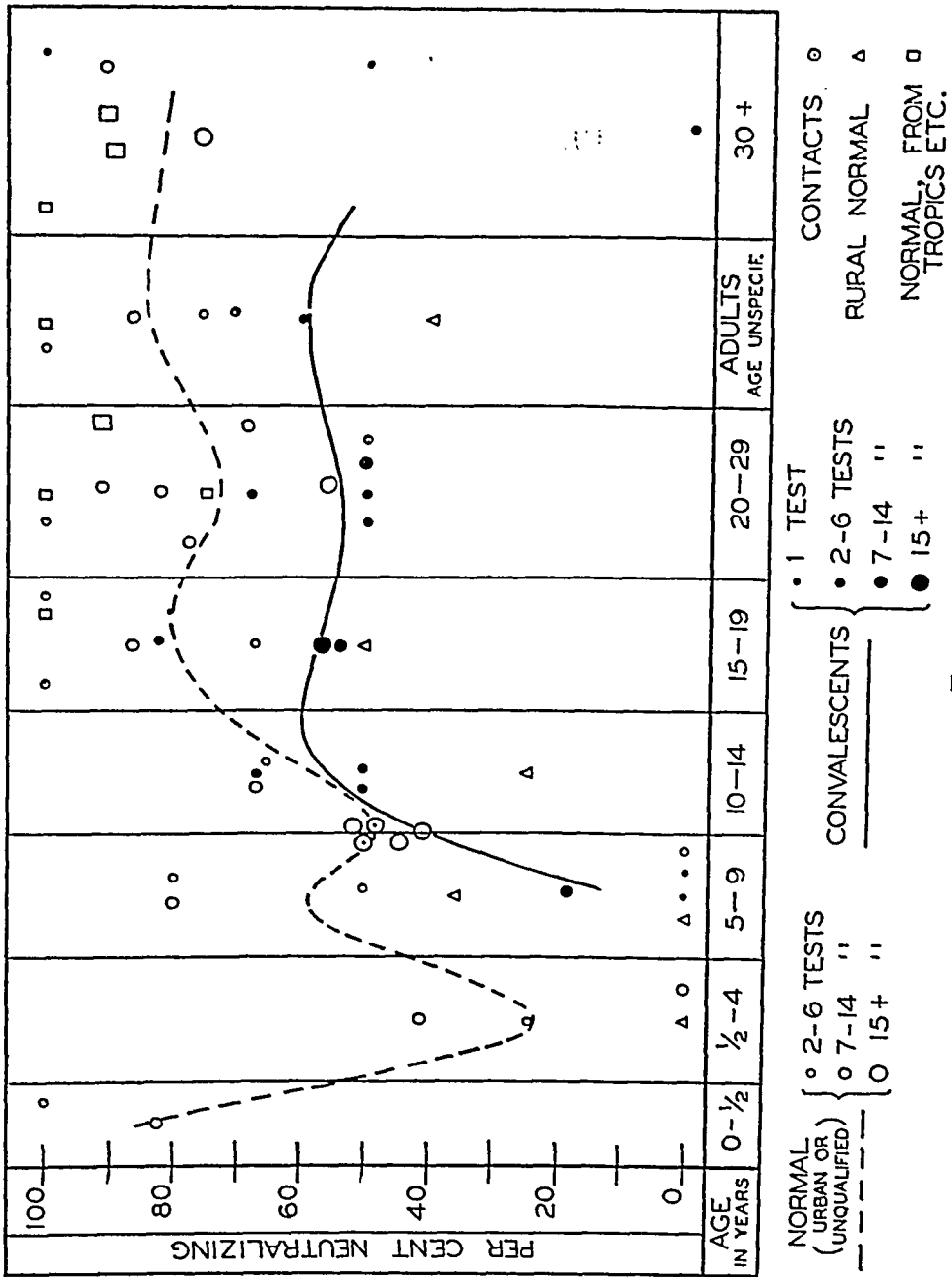


FIG. 1
Figure legend on facing page.

eventually employed for all neutralization tests, using the same dosage as that of the W strain, i.e., a 10 per cent cord suspension mixed with one part of undiluted serum. The F strain was much more virulent for the monkey than our W strain. When mixed with normal monkey serum and inoculated in 0.5 cc. amounts into two monkeys the average incubation period was $3\frac{1}{2}$ days, the febrile period of $2\frac{1}{2}$ days, and the mortality 100 per cent. All of the monkeys which were infected during the course of the neutralization experiments were extensively paralyzed.

Passage Strains

Aycock Strain.—This was obtained through the kindness of Dr. W. L. Aycock of the Department of Preventive Medicine and Hygiene, The Harvard Medical School. It is a highly virulent strain for the monkey. It had been used in several of the investigations (4, 6, 7, 8, 11, 12, 14, 15, 19) referred to in an earlier section of this paper. The number of monkey passages to which it has been sub-

FIG. 1. The per cent of normal, convalescent, and exposed individuals whose sera neutralized passage strains of poliomyelitis virus, arranged according to age groups. Data included in this figure have been obtained from reports (2-20). In several of them (see Bibliography) the published data have been supplemented by a personal letter from one of the authors in which the ages of the individuals tested and other details, not appearing in the text, have been kindly supplied.

Included are the results of tests performed on individuals from: (a) rural districts in this country (shown as small triangles) and from: (b) regions where clinical poliomyelitis is thought to be rare (shown as small squares). Normal represents those individuals from this country, England, and France in whom the environment was not specified as rural. Convalescents represent individuals who in the great majority of instances sustained attacks of poliomyelitis accompanied by residual paralysis. No attempt has been made to separate the convalescent cases into groups depending upon the interval from the time at which they sustained an attack of paralysis and the time when the blood sample was obtained. In those series in which this interval was stated it averaged about 3 or 4 years. Only those individuals have been designated as contacts who had recently (within a few months) been exposed to the disease. The lines which have been drawn through the chart to represent the trends in which positive neutralization tests occurred in normal and convalescent persons, do not include Groups a and b.

Most of the points on the chart have been derived from groups of from 5 to 10 tests performed by a given investigator. A few (three convalescents) represent the results of single tests. Obviously, the result of a single test should not be charted on a percentage basis. They have only been included to aid in the construction of the graph in those age groups in which data on convalescents are scanty.

jected is unknown. After one passage just prior to its use in our experiments (Nov., 1933), a titration of the material showed that 0.5 cc. of a 0.01 per cent suspension would infect a monkey (a smaller dose was not tried). Material from a single animal (No. 1-74) was used for all neutralization tests. The dosage employed was a 1 per cent cord suspension mixed with one part of undiluted serum. When this dose was mixed with an equal part of normal monkey serum and inoculated intracerebrally in 0.5 cc. amounts into three monkeys the average incubation period was 4 days, the febrile period 1½ days, and all three of the monkeys died, as did all other monkeys which contracted the experimental disease in the course of the neutralization tests with this strain.

M Strain.—This highly virulent strain was obtained through the kindness of Drs. W. H. Park and E. R. Weyer from the Bureau of Laboratories, Department of Health, City of New York. It is a mixed strain and so its origin, or the number of monkey passages to which it had been subjected, is unknown. Since we received it in September, 1931, it has had two further passages. Material for all neutralization tests came from a single animal (No. 1-49) and was prepared as a 0.2 per cent suspension, which was mixed with one part of normal monkey serum and injected intracerebrally in 0.5 cc. amounts. The experimental disease in nine monkeys showed an average incubation period of 5 days, a febrile period of 2 days, and a mortality of 100 per cent.

Technique of Neutralization Tests.—This will not be described in detail because the methods employed have been given in a previous publication (25). It may suffice to say that the four strains were employed in the dosages mentioned above, and were mixed with an equal part of undiluted serum in all of our experiments reported in this paper. This mixture was placed in the incubator for 2 hours and monkeys were inoculated intracerebrally in 0.5 cc. amounts. Daily temperature readings were taken over a period of 4 weeks, on all animals thus inoculated. If any animal died within this period from some cause other than experimental poliomyelitis the test was repeated. In the course of the 126 neutralization tests performed in this series of experiments, three such animals died during the 4 week period of observation; two from dysentery, and one from a brain abscess. Individual monkeys were used one to eight times in the course of these experiments which were carried out over a period of a year.

In all experiments in which the neutralizing power of individual samples of human sera were tested, two control tests were employed; a protected control,—in which pooled convalescent serum was mixed with the virus,—and an unprotected control,—in which normal monkey serum was mixed with the virus. If unexplained results occurred in the controls the experiment was discarded. A result of this type occurred once in fourteen experiments. It was an experiment in which the W strain was used, and the protected control developed the experimental disease after a long incubation period. In another experiment, in which the M strain was used, the unprotected control failed to develop the experimental

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disease but this monkey, which was used through an error, was subsequently shown to be resistant to a larger dose of this virus.¹

The results of these tests have been expressed only in terms of a 1:1 serum: virus mixture. A plus sign signifies that neutralizing properties were present in the serum, in that the animal did not develop the experimental disease within the 4 week period of observation; a minus sign signifies that neutralizing properties were absent, in that the animal developed the experimental disease within a few days of the incubation period (to the onset of fever) shown by the unprotected control; and a plus-minus sign signifies so called partial neutralization, in that the incubation period was prolonged for a period of 6 or more days beyond that exhibited by the unprotected control, the longest incubation period noted in all of the experiments being 28 days. A questionable plus sign indicates that three tests were performed and that plus represents the majority result, but that discrepancies occurred in one of the three tests. Protocols of our experiments appear at the end of the paper (Table II). Here it will be seen that thirteen of the passage strain tests were repeated in satisfactory experiments either one or two times. In sixteen repetitive tests discrepancies were encountered twice (\pm as opposed to $+$ once, and $-$ as opposed to $+$ once).

By comparing the results of repetitive tests with those previously reported (25) we find that discrepancies have been noted with the W strain in about 11 per cent of eighteen tests, and with passage strains in about 8 per cent of twenty-five tests. These experiences are mentioned to emphasize that in our hands unknown factors of error in the use of this test are not uncommon, and they reflect to some extent the experience of others (7).

One criticism of the technique of our comparative tests is that different dosages were used for the human strains than were used for the passage strain experiments. Owing to differences in virulence for the monkey exhibited by these strains this has proved a variable which we could not overcome. However, in comparing the human and passage strain tests described in this paper with those

¹ In order to conserve monkeys in the passage strain experiments we frequently used as the unprotected control a monkey which had recovered from a W strain infection. In a previous communication (25) we found that, provided 10 weeks or more had elapsed between the two inoculations, an antecedent infection by the W strain did not protect against an intracerebral inoculation of the M strain, the dose used for the neutralization tests. Reinfection occurred in the six instances in which the experiment was tried. A smaller number of experiments has shown that the Aycock strain will also reinfect. Previously infected monkeys of this type were therefore used not only as unprotected controls, but also as controls in the passage strain neutralization tests (see protocol in Table II). The results of these check tests were only accepted if the monkey subsequently proved to be susceptible.

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which we have previously reported (25) we can find no evidence to show that this variable influences the results in any particular direction.

Sera Employed.—Fourteen samples of normal adult sera obtained from individuals ranging from 21 to 45 years of age, who did not give a history of having had a recognized attack of poliomyelitis, were used in the course of these experiments. These samples were collected in New Haven during the summer of 1931 when poliomyelitis was epidemic in that city.⁴ One specimen was obtained from a physician who had been exposed to poliomyelitis in the course of his hospital duties; the others were from individuals who offered their blood for therapeutic purposes to a member of their family ill with the disease. Two samples of convalescent sera obtained from adults who had sustained attacks of poliomyelitis in childhood, were included as controls in this series. Serum or blood from all of these individuals had been used for therapeutic purposes in 1931, and, as some of the patients so treated had apparently done well and others had done badly it was one of our original objects to attempt to correlate the supposed therapeutic efficacy of the serum or blood with the results of the neutralization tests. The outcome of the tests was such that no such correlation could be made.

Although collected during the summer of 1931 and subsequently kept in the ice box, these sera were not tested until 20 to 32 months had elapsed. It is possible that the neutralizing power of some might have deteriorated between the 20th and 32nd month but no evidence of this supposition was apparent in the course of the experiments. The majority of tests with the passage strains were performed between the 28th and 32nd month. Inasmuch as none of the sera failed to neutralize completely the passage strains of virus, which were the last tests performed, there was no evidence that this particular neutralizing property had been lost by deterioration through aging.

RESULTS

The results of our series of neutralization tests made with four different strains of poliomyelitis virus on fourteen samples of normal (or contact) adult sera and two adult convalescent sera appear in Table I. Comparisons between the different strains may be expressed on a percentage basis if we assume that when partial neutralization is observed with one strain, and either no neutralization or complete neutralization with another, a 50 per cent agreement exists; or, that when questionably positive neutralization is observed with one strain

⁴ Criticism may be applied to the term normal in referring to these individuals. Some of them were intimate contacts. The difficulty of defining the degree of contact in this group was such, however, that it has seemed more practical to consider them all as normal, particularly as we do not believe that the differentiation is pertinent to the problem under investigation.

and positive with another, a 75 per cent agreement exists. On this basis the agreement between the tests with the two human as opposed to the two passage strains might be roughly placed at 75 per cent. On the same basis there is an 86 per cent agreement between the two human strains, and a 92 per cent agreement between the two passage

TABLE I

Neutralization of Normal Adult Sera by Two Human and Two Passage Strains of Poliomyelitis Virus

Subject	Type of individual	Age	Human strains		Passage strains	
			W	F	Aycock	M
R. Hn.	Convalescent	25	+	+	+	+
D. Be.	"	27	+	0	+	+
Epn.	Normal	21	+	+	±	+
D. Bls.	"	26	-	-	0	±
Chn.	"	29	+	+	+	+*
J. Dn.	"	30	+	+	+	+? (c)
Sdb.	"	30	+	+	+	+
Ptg.	"	30	-	+	+*	+*
Klk.	"	34	-	-	+*	+*
Msn.	"	35	+	+	+	+*
Cls.	"	38	+	+	+	+
Wnr.	"	38	+	+	+	+
Brn.	"	40	+	-	+*	+*
Dvl.	"	43	+	+	+	+
Ttr.	"	45	-	-	+	+? (b)
Cpn.	"	45	+	+	+	+*

+, neutralization; ±, partial neutralization; -, no neutralization; 0, test not done (insufficient serum).

+? (a), three tests performed; one -, two +.

+? (b), three tests performed; one ±, two +.

* Test repeated, result confirmed.

strains. The last difference does not appear to be significant if one takes into account the experimental error of the method which we would place at about 10 per cent. It will be seen, however, that in general these normal adult sera showed less power to neutralize human than passage strains. Thus the percentage of positive neutralization

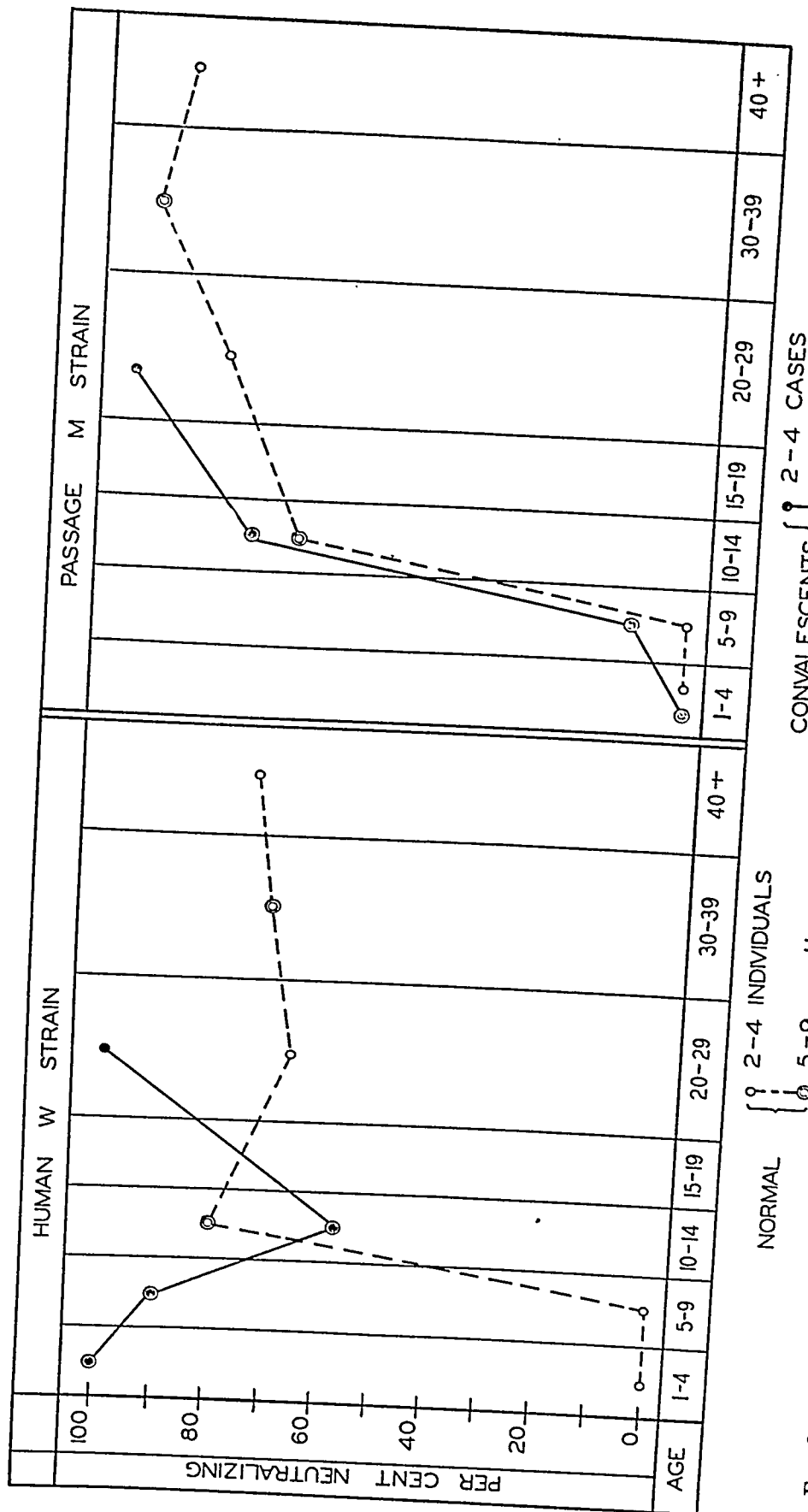


FIG. 2. The results of comparative tests performed on normal persons and patients convalescent from paralytic and abortive poliomyelitis when tested by a human (W) and a passage (M) strain. All of the convalescent sera were obtained within 14 months of the onset of illness. The points in each half of the chart are based on 44 tests, representing 23 normal individuals and 21 convalescent cases.

tests noted with the W strain was 71; with the F strain 71, with the Aycok strain 95, and with the M strain 92.

Our previously reported comparative results were performed on sera from normal children, or children who were in early, late, or convalescent stages of abortive or frank attacks of poliomyelitis. In this younger group, which was largely composed of recent convalescents, not only was there less agreement in the results obtained with the two strains than in the normal adults, but the character of disagreement was generally in the opposite direction; namely, a higher percentage of positive neutralization tests was obtained with the human strain.

The sum total of our results on different types of sera can perhaps be best expressed in terms of graphs which appear in Fig. 2. These have been devised along the same lines as those in Fig. 1. Here it becomes apparent that in our passage strain experiments there is little evidence that the acquisition of either frank or abortive poliomyelitis has any influence upon the results of the tests, and by comparing the right half of Fig. 2 with Fig. 1, it will be noted that our experience with this passage strain has, in general, been similar to those of others with passage strains. With the human strain there is closer agreement with events which one might predict on the basis of analogy with other virus diseases; or in other words, in childhood at least, the acquisition of the clinical disease tends to elevate the "anti-human" viral properties in the blood, although this elevation may not be permanent.⁵

COMMENT

In reviewing our results it should again be pointed out, as has been done in a previous article (25) that many factors are unknown regarding the neutralization test in poliomyelitis. These include: (a) the extent to which a positive test signifies immunity; (b) the time of

⁵ We found in previous work (25) that in testing convalescent sera from mild abortive attacks of poliomyelitis the elevation of "anti-human" viral substance in the blood was occasionally transient. Thus an increase in antiviral substance, demonstrated in two patients in the (3 week) convalescent sample, was not demonstrated in a later (14 month) sample. This transient increase was not noted in several paralytic convalescents similarly tested.

		Human strains														
Strain.....		W—1931, New Haven									F—1931, New York					
Date of experiment.....		May 17, 1933			Sept. 21, 1933			Jan. 30, 1934			Feb. 27, 1934			Apr. 23, 1934		
		Monkey No.	Incubation*	Result	Monkey No.	Incubation*	Result	Monkey No.	Incubation*	Result	Monkey No.	Incubation*	Result	Monkey No.	Incubation*	Result
			days			days			days			days			days	
Control sera	Human convalescent	1-39	+		1-66	12	±	1-94	+		1-83	+		1-88	+	
	Normal monkey	1-52	5	—	1-75	5	—	1-90	7	—	1-84	5	—	1-87	5	—
	" "															
Normal human sera	Brn.	B-19	+													
	Epn.	1-60	+											1-86	8	—
	Klk.	1-44	6	—										2-11		+
	D. Bls.	1-45	5	—										2-03	10	—
														2-04	4	—
	Msn.	1-48	+											1-94		+
	Ptg.	1-47	7	—										1-83		+
	Cpn.				1-71		+	1-83	+		1-86	+				
	Chn.				1-72	9	—	1-84	+		1-85	+				
	Cls.				1-73		+	1-85	+		1-88	+				
	Dvl.				1-74		+	1-86	+		1-87	+				
	J. Dn.				1-76	10	—	1-87	+		1-91	+				
	Sdb.				1-77		+	1-88	+		1-92	+				
	Ttr.				1-78	5	—	1-89	6	—	1-93	6	—			
	Wnr.							1-93	+		1-94	+				
Convalescent sera	R. Hn.				1-48		+	1-91	+					1-91		+
	D. Be.				B-19		+	1-92	+					2-02	Died from abscess	
					Experiment discarded because of result in protected control											
														Preliminary experiment previously infected monkeys		

+, protection; ±, partial protection; —, no protection.

* Incubation period in days to the onset of disease.

† Monkey previously infected with W strain, but susceptible to passage strain.

‡ Monkey previously infected with W strain, subsequently resistant to passage strain.

§ Monkey previously infected with W strain, subsequently resistant to passage strain.

appearance and duration of neutralizing antibodies in the blood after an individual has sustained an attack of the disease; and (c) the specificity of antiviral substance detected by this method. Furthermore, methods of performing neutralization tests in poliomyelitis are not very accurate, and perhaps this criticism can be more justly applied to human than to passage strain experiments. The expense involved, which precludes the performance of large numbers of repetitive tests, and the differences in resistance to infection exhibited by different monkeys, tend to vitiate the results obtained. From the character of the methods employed, therefore, all one can perhaps learn are certain trends or directions in which the results seem to fall. The interpretation of these trends or the clinical or epidemiologic significance of an experiment of this type is dependent, of course, upon the nature of the differences between different types of poliomyelitis virus. This unfortunately is unknown. The implication is, perhaps, that the difference between human and passage strains is comparable to the difference between the street virus as opposed to the fixed virus of rabies; or between yellow fever virus recently isolated from a human case by monkey inoculation as opposed to yellow fever virus which has been adapted to infect mice. However, as far as poliomyelitis virus is concerned, sufficient data do not exist on which to base such an analogy. There is no evidence that all human strains of poliomyelitis virus are alike. There is no evidence that the strains which we and others have designated as human would necessarily acquire the properties of passage strains if they were carried further through five, ten, or twenty monkeys. These and other features go to show that the significance of human *versus* passage strain experiments in poliomyelitis is unknown and that such experiments should be taken at face value and do not warrant wide interpretations.

SUMMARY

In experiments devised to compare the neutralizing action of normal adult human sera on different strains of poliomyelitis virus, and to fill in certain gaps in our series of neutralization tests with different strains of virus on different types of cases in different age groups, we have made the following observations.

1. The difference between two human and two passage strains of

the virus when tested by this method amounted to about 25 per cent, and there was less power in normal adult sera to neutralize human than passage strains of virus.

2. The differences between the two human strains amounted to 15 per cent, and between the two passage strains to 8 per cent, the last figure falling within the limits of the experimental error of the method.

The extent to which these findings affect certain concepts with regard to the epidemiology of poliomyelitis based on passage strain neutralization experiments cannot be determined from the data presented in this paper, except that they more or less confirm the view previously derived from passage strain experiments, that 70 to 95 per cent of normal urban adults possess in their blood, substance which neutralizes poliomyelitis virus in a given amount. However, certain other indications appear when the present results are supplemented by those we have previously obtained (25). Primarily, we have found no relation between the clinical acquisition of poliomyelitis and the presence of substance in the serum which neutralizes a passage strain of poliomyelitis virus. With a passage strain the results seem rather to bear a closer relationship to age than to illness. With a human strain we have obtained results in which there is some evidence, shown only in the juvenile group, that acquisition of the clinical disease is accompanied by the appearance of antiviral properties in the blood.

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THE EFFECT OF VARIOUS PROTEIN RATIIONS ON THE SERUM PROTEIN CONCENTRATION OF THE RAT*

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When rats are placed on a diet low in protein there occurs within a few days a fall of total serum proteins from the control level of approximately 6.25–6.50 gm. per cent to approximately 5.25–5.50. From this point on, however, there appears to be stubborn resistance to further decrease of serum protein concentration, and in experiments previously reported (1) maintenance on a diet almost protein-free for periods of several months did not lead to a further significant drop. These observations suggest that there may be a portion of the blood protein more labile than the rest, which perhaps is readily called on for nutritive purposes when the exogenous supply is cut off. Such a view was supported many years ago by Voit (2) (circulating protein); and the recent observations of Whipple (3) suggest that serum protein introduced intravenously in dogs can to some extent be utilized. At any rate it seemed of interest to study this phenomenon of the "initial" drop of serum protein by examining the effects of various diets with different protein contents.

Methods

Young mature rats (150–200 gm.) were used. In order to simplify conditions the diets were made up of casein and a starch-lard mixture in various proportions. No salts or vitamin preparations were added. The dietary constituents were not, however, chemically purified. Food was offered in abundance and water was not restricted. The animals were weighed at frequent intervals and the total serum proteins were determined on pooled blood (obtained by exsanguination) from groups of four or more rats. The gravimetric method of Barnett, Jones, and Cohn (4) was used.

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In trying to obtain really comparable results with the various diets two difficulties were immediately encountered—the difficulty of time and the difficulty of weight. If, in brief, the rats lost weight uniformly on all the diets and if all the blood protein determinations could be made after the same time interval, then the results would be truly comparable. This was impossible, as a study of Fig. 1 which shows the weight curves demonstrates. On the diet of casein alone, for example, the rats eventually weighed very little less than at the start of the experiment; on all the other diets there was progressive weight loss but at varying rates of speed. It was decided therefore to estimate the proteins in all groups (except the 100 per cent casein group) when a uniform amount of weight loss—approximately 35 per cent of the original—had taken place. This is the maximum loss which rats on any defective diet can tolerate safely; if one pushes the experiment beyond this point certain individuals die even though some can survive further depletion.

It is of interest (see Fig. 1) that speed of weight loss did not vary directly with percentage of protein in the diet when protein was 20 per cent or less. We are not sure that this observation is statistically valid; perhaps with larger groups of rats there would have been a correlation. The point of importance is that with all the smaller protein rations there was very rapid weight loss in contrast to the 50 per cent and 100 per cent casein diets. The exact amount of food taken by each rat could not be measured but since a superabundance was offered it is probable that lack of appetite plays at least a part in the instances of weight loss.

None of the rats on any of the diets showed the usual signs of vitamin deficiency—eye dystrophies, disturbances of hair, diarrhea, etc. There were no gross lesions at autopsy but there was a rough correlation between weight loss and the amount of intra-abdominal fat.

RESULTS

1. *Diet of Casein Alone.*—Thirty-four rats were fed casein alone. Batches of 4–6 were killed on the 6th, 28th, 42d, 98th, 119th, and 140th days. A composite weight curve is shown in Fig. 1. It is seen that the animals lost weight rapidly for about 3 weeks, reached a level, and then gained so that on the 100th day they were only 10 per cent under

the original weight. The total serum protein values are shown in Table I. The net result of prolonged casein feeding, it appears, is that good nutrition and a normal serum protein level are preserved. The initial sharp drop in weight is of interest. It evidently involved some lack of adjustment to the new diet and was associated with a slight fall in the serum protein level from which there was rapid recovery as the animals regained weight.

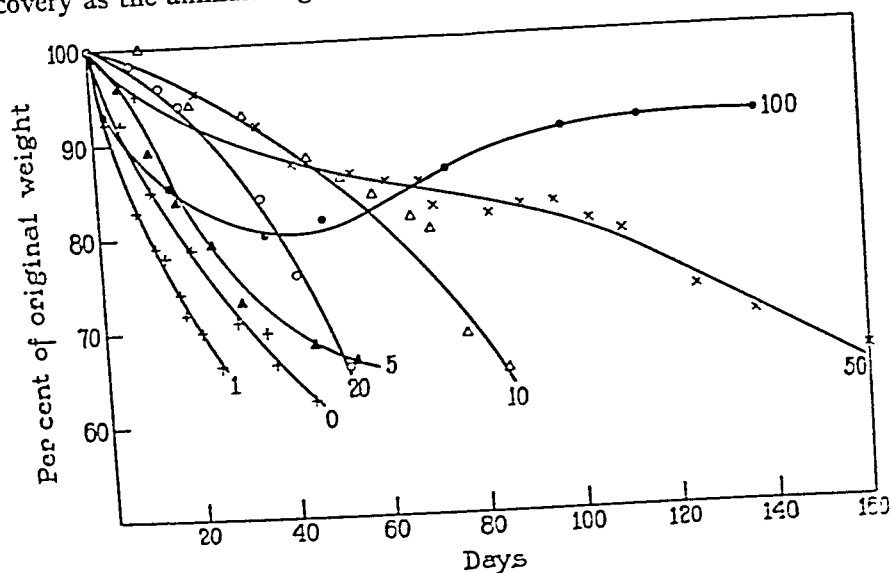


FIG. 1. Composite weight curves of rats on various diets. The numbers at the ends of the curves give percentage of casein in the diet. The remainder consisted of starch-lard mixture.

2. *Diet of Casein 50 Per Cent and Starch-Lard 50 Per Cent.*—Fifteen animals were used. Eight were killed on the 110th day and seven on the 150th day. The serum protein values were respectively 6.17 gm. per cent and 6.24 gm. per cent. The composite weight curve is shown in Fig. 1. Here in contrast to the diet of casein alone there was no sudden initial fall in weight but a steady drop so that at 140 days this group had lost 30 per cent of their initial weight in contrast to the 9 per cent loss of the casein rats. On this regimen, then, as with casein alone there was no significant drop in serum proteins in spite of the fact that the diet was evidently inadequate as evidenced by progressive weight loss.

3. *Diet of Casein 20 Per Cent and Starch-Lard 80 Per Cent.*—Sixteen rats were placed on this diet. As shown by the composite curve in Fig. 1 there was rapid and progressive weight loss. Eight of the animals were killed on the 56th day. Their weight had fallen to 57 per cent of the original value and the serum protein concentration (pooled) was 5.84 gm. per cent. The remaining eight rats were killed on the 63d day. They had not lost as much and still weighed 70 per cent of the original value. The serum proteins were 6.11 gm. per cent. These values for serum protein concentration are only very little below normal and it is evident that even on a diet which leads to rapid loss of weight a relatively small amount of protein suffices to prevent material lowering of blood protein concentration.

TABLE I
Total Serum Proteins of Rats on a Diet of Casein Alone

Batch No.	No. of rats	Length of time on diet	Total serum proteins
		days	gm. per cent
Controls on stock diet			6.25-6.50
A	6	6	6.57
B	6	28	6.07
C	6	42	6.06
D	6	98	6.17
E	6	119	6.25
S	4	140	6.37

4. *Diet of Casein 10 Per Cent and Starch-Lard 90 Per Cent.*—Eight rats were used. The composite weight curve is shown in Fig. 1. On the 85th day the pooled serum proteins were 5.22 gm. per cent. Here for the first time we find a drop to a level definitely below normal. It is not, however, outside the bounds of the "initial" or "physiological" drop which occurs during the first few days on any low protein diet.

5. *Diet of Casein 5 Per Cent and Starch-Lard 95 Per Cent.*—Fifteen rats were used. Ten were killed on the 47th day (weight 67 per cent of initial value) and the serum proteins were 5.46 gm. per cent. Five were killed on the 53d day (weight 66 per cent of initial value) with serum proteins 5.55 gm. per cent. These values are definitely not

below those which result from the initial drop, and it should be noted that lowering the protein content of the diet from 10 per cent to 5 per cent led to no further fall in serum protein values.

6. *Diet of Casein 1 Per Cent and Starch-Lard 99 Per Cent.*—Ten rats were placed on this diet. As shown in Fig. 1 there was rapid weight loss so that by the 25th day weight was reduced to 66 per cent of the initial value and it was thought advisable to sacrifice the animals. The serum proteins were 5.59 gm. per cent, a value, as it happens,

TABLE II
Summary of Effect of Various Rations on Serum Protein Level

Diet	Duration of experiment	Loss of weight	Serum protein concentration
			gm. per cent
Casein alone	120-140	10±	6.25-6.37
Casein 50% Starch-lard 50%	150	33	6.25
Casein 20% Starch-lard 80%	63	30	6.11
Casein 10% Starch-lard 90%	92	36	5.46
Casein 5% Starch-lard 95%	53	34	5.55
Casein 1% Starch-lard 99%	25	34	5.59

higher than that obtained in the rats which received 5 and 10 per cent of casein.

7. *Diet of Starch and Lard; No Casein.*—We have reported at length studies of the effect of this diet on serum proteins (5). There is the usual initial drop often followed by no further lowering of protein although in some groups of rats which have been carried for 5-7 weeks we have obtained values of from 4.50-5.00 gm. per cent. This diet, however, with no proteins, vitamins, or salts, subjects the animal to a drastic attack on its nutrition.

The results of all the experiments are summarized in Table II.

DISCUSSION

To make clear the significance of these experiments: We have previously demonstrated that on low protein diets there is a prompt (few days) fall of serum protein concentration in the rat from a control level of 6.25–6.50 to a level of 5.25–5.50 gm. per cent. The drop amounts roughly to about 20 per cent. Thereafter, even if the low protein diet is continued for long periods there may be no further decline of serum protein concentration. The object of the present experiments was to determine with successive decreases of protein in the diet at what point the phenomenon of the initial drop took place.

It appears that with protein rations of 50 per cent or over there was no drop at all in the concentration of serum proteins. With the 20 per cent casein diet there was a very slight lowering of questionable significance. With protein rations under 20 per cent there was no further fall after the initial drop of approximately 1 gm. per cent regardless of whether protein made up 10, 5, or 1 per cent of the diet.

These experiments seem in accord with the view that a certain fraction of the serum proteins can be called on for metabolic purposes when the intake is inadequate. When this fraction has been disposed of, however, further fall of blood protein concentration can be achieved only by drastic procedures as if there were a resistance to alteration of the fundamental structure of blood colloids just as there is to alterations of acid-base equilibrium.

The following additional experiments are compatible with the views expressed above.

Sudden Changes from High to Low Protein Diet.—Eight rats were placed on a diet of casein alone. They were divided into two lots of four each. On the 136th day the weight of Lot 1 was 89 per cent and of Lot 2, 96 per cent of the original value. On this day Lot 2 were changed from a diet of casein alone to a diet of starch and lard with no casein; Lot 1 continued on casein alone. 48 hours later both lots were bled. The pooled serum proteins of Lot 1 were 6.37 gm. per cent, of Lot 2, 5.44 gm. per cent. In brief, in Lot 2 even though the animals were on an excessively high protein ration for 136 days, a 2 day change to starch-lard led to a sudden drop of blood proteins of the type which we have described. During the final 2 days of the experiment,

Lot 1 gained from 89 per cent of original weight to 91 per cent, whereas Lot 2 gained from 96 per cent to 100 per cent even though the serum protein fell. This gain was probably partly due to water retention.

Sudden Change from Low to High Protein Diet.—This experiment, reported in detail elsewhere (1), was in brief as follows: Ten rats were placed on a diet practically free of protein but otherwise adequate. They were divided into two lots of five each. On the 140th day Lot 1 were bled and the serum protein concentration was 5.14 gm. per cent. Lot 2 were then placed on a stock diet containing 16 per cent of casein and 48 hours later they had gained from 87 per cent to 95 per cent of original weight. On bleeding, the serum proteins were 6.19 gm. per cent. The result is exactly the reverse of the preceding experiment.

These very rapid changes in blood protein obtainable in animals previously stabilized over long periods of time on high or low protein diets seem compatible with the view that there is a labile portion of the blood proteins apart from the basic blood colloids.

SUMMARY

1. The effect of various percentages of protein (casein) in the diet on the serum protein concentration of the rat was studied.
2. With high protein rations (50 per cent or over) there is no drop at all of serum protein concentration, even though the diet is otherwise poorly balanced and inadequate as evidenced by weight loss.
3. With protein rations under 20 per cent the serum protein concentration falls promptly to a level of about 5.25–5.50 gm. per cent, where it is maintained regardless of whether the diet contains 1, 5, or 10 per cent of protein. Further depletion of the blood colloid concentration seems to meet with resistance on the part of the body.

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FURTHER STUDIES WITH TOXIC SERUM EXTRACTS OF HEMOLYTIC STREPTOCOCCI

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In a previous paper¹ it was shown that under certain definite conditions serum extracts of hemolytic streptococci are markedly hemolytic and leucocidic and are toxic for mice when injected intravenously, causing hemoglobinuria, anemia, and death. The present paper deals with further work carried on with these toxic extracts.

EXPERIMENTAL

Preparation, Preservation, and Toxicity of the Toxic Serum Extracts of Streptococci.—

The method used for the preparation of the toxic extracts was the same as that previously described. In this work only horse serum was used for the extractions. In our earlier paper we noted that in the preparation of the toxic extracts, the same streptococci could be subjected to extraction three times with untreated, inactivated serum without loss of potency in the later extracts if all three extractions were carried out on the same day. However, any additional extractions carried out with these same organisms that had been kept overnight in the ice box resulted only in preparations weak in hemotoxin and innocuous for mice. Subsequently, we found that if streptococci subjected to extraction three times on 1 day were kept frozen solid over that night, they could still be resubjected to extraction three times on the 2nd day without loss of potency in the fourth, fifth, or sixth extracts. This process, however, when repeated on the 3rd day resulted in only weak preparations.

As to the preservation of the serum extract poisons, we stated previously that they deteriorate quickly even at ice box temperatures though covered with vaseline seals. This great lability of the poisons necessitated the preparation of fresh extracts for each day. Of great advantage was the later finding that the toxic extracts when kept frozen solid may be preserved without deterioration for at least 6 months.

¹ Weld, J. T., *J. Exp. Med.*, 1934, 59, 83.

In our previous work the most potent toxins obtained were those that in 0.1 cc. amounts killed 18 to 20 gm. mice in one day. Subsequently, no extracts more toxic than these have been produced, although we have tried to obtain them in many different ways. It would appear, therefore, that serum can absorb only a certain definite amount of toxin from the streptococci and at least for this strain of streptococcus, the serum is saturated when it contains approximately ten lethal doses of toxin per cc. for mice. That the organisms themselves contain a much greater quantity of toxin than this is evident from the fact stated above, that under certain conditions they can be subjected to re-extraction six times with no loss of toxicity in the last extracts. Moreover, it seems likely that more than six extractions could be accomplished without weakening of the later extracts, were it not for the slow deterioration of the toxin on the organisms which must occur because of unavoidable exposure to the harmful influence of temperatures much above 0°C. during the many manipulations. All of this evidence seems to demonstrate how potentially toxic are the cells of *Streptococcus hemolyticus*.

Concentration of the Toxic Extracts.—We found that $\frac{1}{2}$ saturation with $(\text{NH}_4)_2\text{SO}_4$ precipitated out practically all of the hemotoxin from a toxic extract, whereas normal inactivated serum treated in the same way was not hemolytic even in relatively large amounts. In regard to the toxic properties of $(\text{NH}_4)_2\text{SO}_4$ -precipitated toxin for mice, we have no data since the amount of $(\text{NH}_4)_2\text{SO}_4$ these preparations contain was sufficient to be toxic in itself, and dialyzing a preparation to rid it of $(\text{NH}_4)_2\text{SO}_4$ destroyed its toxic properties both for red cells and for mice.²

Toxicity of Serum Extracts of Hemolytic Streptococci Produced from Strains Other than That Originally Used (Gay) and Possible Correlation between the Toxicity of an Extract and the Virulence of the Organisms from Which It Was Produced.—In our previous work, only one strain of *Streptococcus hemolyticus* was used for the preparation of the serum extract poisons. Therefore it seemed of interest to determine whether similar poisons could be obtained from other strains of hemolytic streptococci and whether the virulence of any one strain was related to its ability to produce a toxic serum extract. Accordingly, serum extract filtrates were prepared from a number of strains of *Streptococcus hemolyticus* obtained from various sources by the same method

² We are indebted to Dr. Henry S. Simms for dialyzing a $(\text{NH}_4)_2\text{SO}_4$ -precipitated toxin for us in his iced apparatus (Simms, H. S., *J. Exp. Med.*, 1930, 51, 319).

as that used previously. Then these filtrates were titrated for their killing properties for mice and at the same time the virulence of the various organisms was worked out. The filtrates were also titrated for hemotoxins to see whether the hemotoxin content of a preparation was related either to the killing properties of a toxin or to the virulence of the strain from which the toxin had been made.

Table I gives the results of this experiment,³ and seems to indicate that there is no correlation between the virulence of an organism for mice and its ability to produce poisonous serum extracts. Although it is true that the Gay and C203 (51st and 52nd passages), the two most virulent strains, are also the best toxin producers, still even with these two strains there is no proportionate correlation between the two factors mentioned, since they both produce the same strength toxin and yet the C203 strain is 100 times as virulent as the Gay strain. In regard to the other strains, all of which were much less virulent than the Gay strain, there was even less correspondence between the two factors—for instance, some strains were avirulent and produced toxin, see Strains 50 and 83, while others were virulent and produced no toxin, see Strains 65 and NY5. In our previous paper it was pointed out that there appeared to be no correlation between the hemolytic and lethal properties of a serum extract toxin produced from the Gay strain. This statement appears to apply also to the other strains worked out here (see Table I).

The Pathology of Mice Dying of the Streptococcus Serum Extract Toxin.—The pathology of eight mice dying after the inoculation of the toxin from the Gay strain was studied by Dr. Maurice N. Richter.

³ The sources of the strains of *Streptococcus hemolyticus* used in the table were as follows:

Gay.—Erysipelas strain. The same strain as that used in our earlier work and in most of this work. Kept virulent by passage through rabbits.

C203 (51 and 52).—Rockefeller strain, 51st and 52nd passages through mice obtained from Mr. C. M. Soo Hoo.

C203 (Mr. Ma, and G).—Matt virulent, matt avirulent, and glossy, respectively and Starr spinal fluid strain, obtained from Miss Miriam Olmstead.

R65.—Toxic rheumatic strain.

NY5.—Dochter scarlet fever strain and rheumatic Strains 50, 57, 67, 83, Aronson, 46, 47, 90, and 38, obtained from Miss Ruth Pauli.

Briefly, all showed marked degenerative changes in the tubular epithelium of the kidney which consisted in loss of nuclear staining and desquamation of cells and colloid or hyaline in the form of droplets

TABLE I

Relationship between the Toxicity of an Extract and the Virulence of the Organisms from Which It Was Produced

Strain	Mouse-killing dose of extract	Hemolytic unit	Virulence
	cc.	cc.	
Gay	0.2	0.002	10^{-5}
C203 (51)	0.2	0.003	10^{-7}
C203 (52)	0.2	0.003	10^{-7}
C203 Mv	0.5	0.008	10^{-7}
C203 Ma	0.4	0.006	10^{-4}
C203 G	0*	0.02	0†
Loeffleman	0.5	0.005	10^{-2}
50	0.5	0.005	0†
57	0.5	0.005	10^{-1}
67	0.5	0.003	10^{-1}
83	0.5	0.003	0†
R65	0*	0.005	10^{-2}
NY5	0*	0.005	10^{-2}
Aronson	0*	0‡	10^{-1}
Starr	0*	0.07	10^{-1}
46	0*	0.005	10^{-1}
47	0*	0.02	0†
90	0*	0.07	10^{-1}
38	0*	0.2	0†

From the Gay strain and from all the C203 variants, at least two different extracts were made and these were titrated separately for hemotoxic and mouse-killing units. In the table, the average of these results is noted for each strain.

* 0.5 cc. of the serum extract filtrate did not kill a mouse.

† 0.5 cc. of 1-10 broth culture of the streptococcus did not kill a mouse when injected intraperitoneally.

‡ 0.1 cc. of the serum extract produced no hemolysis.

in the tubular epithelium. The glomeruli showed no changes. In six of the eight mice there was marked necrosis of the lymphoid cells in the follicles of the spleen and in two instances in the pulp as well. In four mice there were many small focal necroses in the liver.

SUMMARY

1. The same *Streptococcus hemolyticus* organisms may be subjected to extraction six times in 2 days with untreated inactivated serum with no loss in potency of the later extracts when the organisms are kept frozen solid during the night between the extractions.
2. The serum extract toxins of hemolytic streptococci can be preserved without deterioration for at least 6 months if kept frozen solid.
3. No toxins stronger than those containing 10 units per cc. for mice have been prepared. Reasons for thinking that this is due to the saturation of the serum with the toxin at this point are given.
4. Half saturation with $(\text{NH}_4)_2\text{SO}_4$ precipitates out practically all of the hemotoxin in a preparation.
5. Serum extracts were made from strains of hemolytic streptococci other than the Gay strain and attempts were made to correlate the virulence and toxin production from each strain. No such correlation could be established.
6. The principal pathologic finding in mice inoculated with the streptococcus serum extract toxin is a marked degeneration of the tubular epithelium of the kidney.

EXPERIMENTAL STUDIES ON ENCEPHALITIS

III. SURVIVAL OF ENCEPHALITIS VIRUS (ST. LOUIS TYPE) IN ANOPHELES QUADRIMACULATUS

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Experiments on the mode of transmission of encephalitis (St. Louis type) have dealt thus far with the theories of direct person to person spread by way of the upper respiratory tract and spread by the bite of infected mosquitoes. Favoring the first idea are the experiments with mice demonstrating that virus instilled into the nose in small quantities readily gives rise to encephalitis (1), but that virus injected into the blood stream directly or indirectly is relatively innocuous (2). Moreover, mice receiving sublethal doses of virus intranasally are reported to become immune to subsequent lethal doses (3), and persons in close contact with human cases of encephalitis are said to show specific protective properties in their sera (4). Finally, mosquitoes fed directly upon human patients ill with encephalitis and then allowed to bite healthy persons failed to transmit the disease (5). Although these observations favor the theory of person-to-person spread of this encephalitis by way of the upper respiratory tract, the puzzling fact remains that the seasonal incidence of the disease, August and September, corresponds in general, not with that of respiratory tract infection but rather with that which is borne by food, water, or insects.

One step in the analysis of the hypothesis of an insect vector has been made by determining whether the virus will survive in the mosquito host. In a single test with *Aedes aegypti*, filtrates of infected mosquitoes became non-infective within 3 days; in several tests with

Anopheles quadrimaculatus, on the contrary, the virus survived. The experiments were carried out in the following manner.

Technique

Brains from at least five mice dying 4 to 5 days after an intracerebral injection of mouse brain virus were removed and pooled, ground with alundum, suspended in equal parts of hormone broth and ascitic fluid, and diluted one part by weight of brain in nine parts volume of fluid. This suspension was centrifuged at 3000 R.P.M. for 30 minutes and 1.5 cc. injected intraperitoneally into Swiss mice. Virus so injected is known to be present in the blood stream for at least 5 hours in amounts exceeding 5000 fatal intracerebral doses per cc. The injected mice were immobilized by tying to boards, and within 60 minutes after receiving the virus were placed with abdomens in contact with the gauze tops of cages containing starved mosquitoes and left there under observation from 1 to 2 hours. They were then released and placed in cages for observation.

The mosquitoes employed were raised in the Laboratories of the International Health Division of the Rockefeller Foundation by Mr. Thomas L. Cain, Jr. (6). Lots of 200 to 400 females, 4 to 7 days old, were used in each test. A given lot was allowed to feed on infected mouse blood for 1 to 2 hours, as described above, and those not feeding were discarded. Within 4 hours of feeding, ten engorged mosquitoes were selected at random, killed with tobacco smoke, ground in a mortar, diluted with 3 cc. of equal parts of ascitic fluid and hormone broth. The suspension was centrifuged at 2500 R.P.M. for 20 minutes and then filtered through a Seitz pad 2.4 cm. in diameter, at 30 pounds pressure. The filter was previously saturated with protein by passing 10 cc. of hormone broth through it at 15 pounds of pressure. This procedure permits passage of the virus with no appreciable loss (7). Tenfold serial dilutions were made of the filtrate and each was injected intracerebrally in 0.03 cc. amounts into four to six Swiss mice. The injected animals were observed 21 days and their duration of life recorded.

The test lot of mosquitoes was maintained according to the routine laboratory procedure. At varying intervals, samples of ten were tested as described for detecting quantitatively the presence of virus. Virus recovered from the final sample from a given lot of mosquitoes was shown to be identical immunologically with the original test virus.

Results of Titrations

Aedes aegypti.—One test was made with *Aedes aegypti*. As shown in Table I, the virus was present in the mosquitoes 4 hours after feeding on the infected mice. All mice receiving undiluted filtrate and filtrate diluted 10^{-1} died, and two of the six receiving the 10^{-2} dilution died. If the titration end-point is taken as the dilution killing 50

per cent or more of the mice (8), the quantity of infective virus per mosquito at 4 hours may be estimated as follows: Taking the end-point as the 10^{-1} , or 0.03 cc. of 10^{-1} dilution, there must have been 1000 mouse lethal doses in the 3 cc. of filtrate, or 1000 lethal doses in ten mosquitoes and 100 lethal doses in each mosquito.

At 3, 5, 8, 11, 14, and 20 days after feeding, samples of ten of the lot were tested but failed to kill the mice. No further tests on *Aedes aegypti* could be made at this time; hence this result is given merely as a matter of record.

TABLE I
Lack of Survival of Encephalitis Virus (St. Louis Type) in Aedes aegypti

10 mosquitoes tested	Interval feeding to testing	Dilution of mosquito filtrate (6 mice per dilution)			
		Undiluted	10^{-1}	10^{-2}	10^{-3}
		Duration of life			
	hrs.	days	days	days	days
1934					
Jan. 15	4	5, 5, 5, 5, 5, 6	5, 6, 6, 6, 6, 9	6, 6	9
" 18	3				
" 20	5				
" 23	8				
" 26	11				
" 29	14				
Feb. 5	20				

Blank spaces indicate mice surviving and well 21 days.

Anopheles quadrimaculatus.—Six experiments were made with *Anopheles quadrimaculatus*. In each experiment, samples of mosquitoes tested within 4 hours of feeding on the infected mice contained demonstrable virus. Samples tested 2 to 15 days later showed less virus than was demonstrated in the first sample. Samples tested after 25 days showed virus present in amounts approximately equal to but not exceeding the titre in the original sample.

Experiment 1, April 2, 1934.—Female *Anopheles quadrimaculatus* mosquitoes were permitted to feed 2 hours on mice which had received 1.5 cc. of a 1 to 10 emulsion of mouse brain virus. Those shown to be engorged were saved; the remainder were discarded. 4 hours after feeding, ten mosquitoes were killed and

titrated as far as 10^{-3} . Table II shows that all mice receiving the undiluted 10^{-1} , 10^{-2} , and 10^{-3} suspensions died, indicating that at this time each mosquito contained at least 10,000 mouse lethal doses of virus. 3 days later, the titre had dropped to 100 lethal doses per mosquito. On the 7th day, the titre was between 10 and 100, on the 10th and 14th days, 100 lethal doses, on the 17th and 21st days, 1000 lethal doses, on the 24th day 10,000, and on the 30th day, 100,000 lethal doses per mosquito. This final titre of 100,000 could not be regarded as greater than the titre of at least 10,000 in the original sample of infected mosquitoes since in that sample dilutions of 10^{-4} and greater were not tested.

TABLE II

Survival of Encephalitis Virus (St. Louis Type) in Anopheles quadrimaculatus Experiment 1

10 mos- quitoes tested	Interval feeding to testing	Dilution of mosquito filtrate (6 mice per dilution)				
		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}
		Duration of life				
1934	hrs.	days	days	days	days	days
Apr. 2	4	4, 4, 4, 5, 5, 5	5, 5, 5, 6, 6, 6	5, 5, 5, 5, 5, 6	5, 6, 7, 7, 7, 8	—
	days			—	—	—
" 5	3	6, 6, 6, 7, 7, 8	6, 7, 7, 7, 7, 8			—
" 9	7	5, 5, 5, 6, 7	8			—
" 12	10	6, 6, 7, 7, 7, 8	6, 7, 7, 7, 7, 8			—
" 16	14	5, 5, 5, 5, 5, 6	5, 5, 5, 5, 6, 6	6, 6		—
" 19	17	5, 5, 6, 6, 7, 7	6, 7	7, 7, 7		—
" 23	21	6, 6, 6, 7, 7, 7	6, 6, 7, 7, 7, 8	6, 6, 6, 7, 7, 7	7	—
" 26	24	4, 5, 5, 5, 5, 5	5, 5, 5, 5, 6, 6	5, 5, 5, 5, 6, 6	5, 6, 6, 6	—
May 2	30	5, 5, 5, 5, 5, 6	5, 5, 5, 5, 6	5, 6, 6, 6, 6	5, 6, 6	5, 5, 6

Blank spaces indicate mice surviving and well 21 days. Dashes indicate given dilution not titrated.

Experiment 2, June 19, 1934.—252 engorged female mosquitoes were tested as in Experiment 1. The titre of samples tested 4 hours after feeding was 10,000 lethal doses. This dropped to a titre of 10 lethal doses on the 3rd day, and increased to 100 on the 14th and 24th days (Text-fig. 1). An unusually high mortality among the mosquitoes terminated this experiment before the final titrations could be run.

Experiment 3, July 6, 1934.—The same experiment was repeated a third time with 207 mosquitoes which fed on infected mice (Table III). The immediate titre of the 4 hour sample of ten mosquitoes was 10,000 lethal doses per mosquito. On the 4th day it had dropped to 100; on the 7th day to less than 100; on the 11th

and 14th days it had increased to 1000; and on the 18th day to 10,000. On the 25th day it was 1000, on the 33rd day 100, and on the 42nd day 100,000. These last three titrations are irregular and cannot be taken to indicate that the titre of the final sample surpassed that of the freshly fed mosquitoes.

Experiment 4, October 19, 1934.—The test was repeated a fourth time on 168 female mosquitoes fed on infected mice, with more regular results (Table IV). The titre of virus in the 4 hour sample was the same as previously, 10,000 lethal mouse intracerebral doses per mosquito. On the 4th day the titre had dropped to 1000; on the 7th and 11th days to 100 and 10, respectively. It had increased

TABLE III
Survival of Encephalitis Virus (St. Louis Type) in Anopheles quadrimaculatus
Experiment 3

10 mos- quitoes tested	Interval feeding to testing	Dilution of mosquito filtrate (6 mice per dilution)				
		Undiluted	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
		Duration of life				
1934	hrs.	days	days	days	days	days
July 6	4	6, 6, 6, 6, 6, 6	6, 6, 6, 6, 6, 7	6, 6, 6, 7, 8, 8	10, 10, 12, 13	12, 13
" 10	4	6, 7, 8, 8, 10	7, 7, 8, 8	7		
" 13	7	6, 6, 7, 7, 8	7, 8			
" 17	11	6, 6, 6, 6, 7, 10	6, 6, 10, 13	9, 9, 10		6, 6
" 20	14	5, 6, 6, 7, 9, 10	7, 10, 10, 10, 10	5, 8, 8, 9	7, 8	11
" 24	18	6, 7, 7, 7, 9, 9	6, 7, 7, 8, 9	7, 8, 8, 10, 10	7, 7, 7, 8	7, 7
" 31	25	6, 6, 6, 6, 6, 6	5, 6, 6	6, 7, 10	12	
Aug. 8	33	5, 5, 5, 5, 6, 6	5, 6, 6, 6, 7, 7	7		
" 17	42	5, 6, 7, 7, 7	6, 6, 6, 6, 7, 7	6, 7, 7, 7, 7	8, 8, 8	9, 9, 9

Blank spaces indicate mice surviving and well 21 days.

to 10,000 on the 19th day and continued at the level of the original titre on the 26th and 33rd days.

Experiment 5, November 1, 1934.—In the previous experiments no evidence of multiplication of virus in the mosquito had been obtained since the titres of virus in mosquitoes 4 hours after feeding were not surpassed at any later period. In Experiment 5, smaller amounts of virus were injected into the mice as possibly a more sensitive method of detecting an increase above the original low titres.

Lots of twenty-five mosquitoes were each fed on mice given an intraperitoneal injection of 0.5 cc. of mouse brain virus in dilutions of 1 to 10, 1 to 100, 1 to 400, and 1 to 1600. A titration of virus in a sample of ten mosquitoes was made 4 hours after feeding and in the survivors 27 days later. The results are shown in Table V. Lot A, fed on mosquitoes given the same approximate amount of virus

TABLE IV
Survival of Encephalitis Virus (St. Louis Type) in Anopheles quadrimaculatus
 Experiment 4

10 mosqui- toes tested	Interval feeding to testing	Dilution of mosquito filtrate (4 to 6 mice per dilution)									
		No. of mice injected	Undiluted	No. of mice injected	10 ⁻¹	No. of mice injected	10 ⁻²	No. of mice injected	10 ⁻³	No. of mice injected	10 ⁻⁴
			Duration of life		Duration of life		Duration of life		Duration of life		Duration of life
			days		days		days		days		days
1934	hrs.										
Oct. 19	4	6	5, 5, 5, 5, 6, 6	6	5, 5, 5, 5, 6, 6	6	5, 6, 6, 6, 6, 7	6	6, 6, 6, 7	6	
"	days										
" 23	4	4	6, 7, 7, 7	4	6, 7, 9, 9	6	6, 6, 10, 11	6		4	
" 26	7	4	6, 6, 7, 8	4	6, 6, 7, 7	6	6, 8	6		4	
" 30	11	3	6, 6, 6, —	3	8, 9, —	6	9	6		4	
Nov. 7	19	3	5, 6, 7, —	4	5, 6, 6, 9	6	5, 5, 6, 8	6	6, 9, 10	4	
" 14	26	4	5, 6, 6, 7	4	6, 6, 6, 7	6	6, 7, 7, 7, 8	6	6, 7, 7, 8, 9, 10	4	
" 27	33	5	6, 6, 6, 6, 6	5	7, 7, 7, 8, 9	5	8, 8, 9, 9, 10	5	9, 9, 9, 10, 11	4	

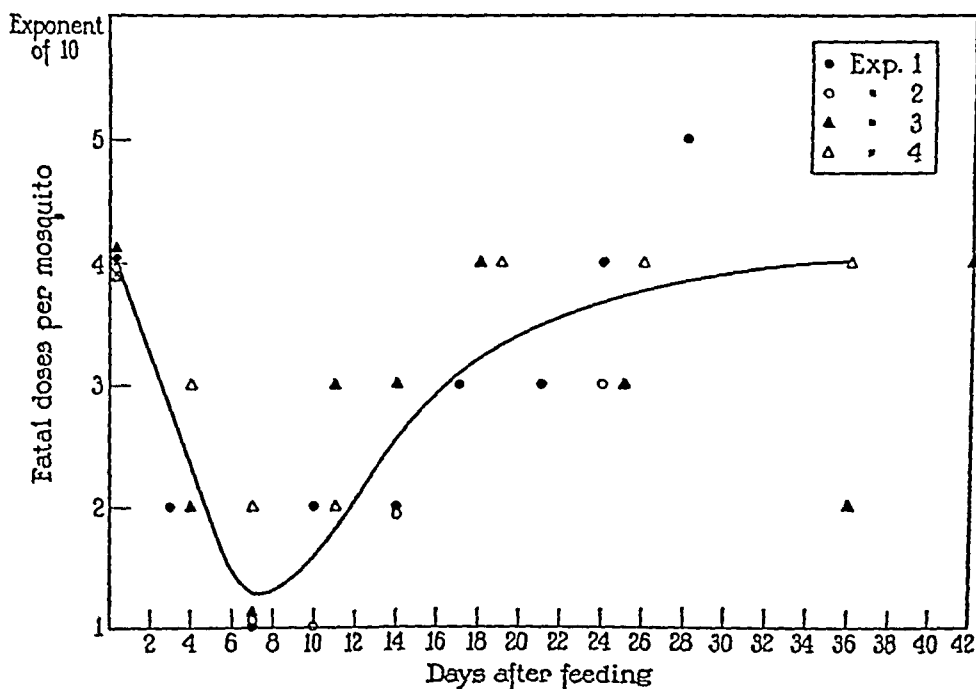
Blank spaces indicate mice surviving and well 21 days.

TABLE V
Survival of Encephalitis Virus (St. Louis Type) in *Anopheles quadrimaculatus*
Experiment 5

Mosquito lot	Dose of virus per mouse, cc.	10 mosquitoes tested	Interval feeling to testing	Dilution of mosquito filtrate (6 mice per dilution)				
				Undiluted	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
				Duration of life				
A	1:10	Nov. 1	4 hrs.	days	days	days	days	days
	1:10	" 28	27 days	6, 6, 6, 6, 6	6, 6, 6, 6, 7	6, 6, 6, 7, 7	6, 7, 7, 7	7
B	1:100	" 1	4 hrs.	5, 5, 5, 5, 5	5, 5, 5, 6, 6	5, 6, 6, 6, 6	5, 6, 6, 8, 8	8
	1:100	" 28	27 days	6, 6, 6, 6, 7	6, 6, 7, 7, 8	6, 6, 6, 6, 6	5, 6, 6, 8, 8	
C	1:100	" 1	4 hrs.	7, 7, 7, 7	6, 7, 8			
	1:100	" 28	27 days	6, 6, 7, 7				
D	1:1000	" 1	4 hrs.	6, 6, 6, 7	6, 7			
	1:1000	" 28	27 days	5, 5, 6, 6	7, 7			

Blank spaces indicate mice surviving and well 21 days.

as in the previous four experiments, showed the characteristic immediate and 27 day titres; namely, 10,000 lethal doses per mosquito. Lot B, fed on mice given 1/10 of this dose, showed an immediate titre of 100 and a final titre of 10 lethal doses. Lot C, fed on mice given 1/40 of the standard amount, showed an immediate titre of 100 lethal doses and no virus on the 27th day. Lot D, fed on mice given 1/160 of the standard dose, showed similar immediate and final titres of 100 lethal doses. No evidence of multiplication of virus was obtained in this test.



TEXT-FIG. 1. Titration of encephalitis virus (St. Louis type) in *Anopheles quadrimaculatus* mosquitoes fed on infected mice.

Failure of Virus-Containing Anopheles quadrimaculatus Mosquitoes to Transmit the Infection to Mice and Monkeys

Virus-containing *Anopheles* mosquitoes were tested for their ability to transmit the infection to mice and monkeys, although these animals were known to be resistant to experimental blood stream infection (2). At various intervals after engorging on infected mice, lots of mosquitoes were fed on normal mice and monkeys. None of these contracted encephalitis or developed immunity or serum-neutralizing substances against the virus.

DISCUSSION

The experiments described are believed to constitute the first instance in which an *Anopheles* mosquito has been shown to harbor a virus. The encephalitis virus in *Anopheles quadrimaculatus*, unlike yellow fever virus in *Aedes aegypti* (8), has been shown to be present 21 to 42 days in quantities approximately equal to that originally ingested. The original titre was not exceeded, however, even in instances in which it was relatively low. Hence the virus cannot be said to have multiplied in this insect host.

Knowledge that the virus of encephalitis can persist in a species of mosquito prevalent in regions in which the disease occurs is of basic importance in testing the insect transmission theory.

CONCLUSIONS

Anopheles quadrimaculatus mosquitoes, fed on mice in which encephalitis virus (St. Louis type) is present in the blood stream, take up and retain the virus for the duration of their lives.

The titre of the virus in mosquitoes 4 hours after engorging on mice with a maximum blood stream infection represents about 10,000 lethal mouse intracerebral doses per mosquito. This titre drops during the following 2 weeks to about 100 lethal doses per mosquito, but from the 3rd week to the death of the mosquito, usually increases to approximately the original level and remains there.

The titre of virus in mosquitoes which have engorged on mouse blood containing smaller quantities of virus exhibits the same drop and subsequent rise to the original level.

The virus-containing mosquitoes did not infect mice or monkeys by biting.

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PRIMARY SERUM TOXICITY AS DEMONSTRATED BY THE CHICKEN EMBRYO

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The origin of normal antibodies for bacteria and their toxins is still a moot question. There is, however, no doubt that antibody functions of normal sera against such antigens as blood group properties or Forssman antigen are due to constitutional, genetic factors. Normal sera containing Forssman antibodies react with cells and tissues which possess Forssman antigen; that is to say, such sera can display "primary toxicity."

Szepesnwol and Witebsky (1, 2) have recently shown that from the beginning of its development the chicken embryo, as well as its vascular network, contains Forssman antigen. Addition of normal human serum, rabbit serum or Forssman antisera to the chicken embryo causes a peculiar phenomenon. The vascular network shrinks, the embryo turns around and sinks into the yolk. One can often see the heart beating within the sunken embryo, until it finally dies¹ (Baumann and Witebsky (3, 4)). Duck embryos, lacking Forssman antigen, however, do not show the vascular phenomenon after the addition of normal human or rabbit serum. The experiments to be reported in this paper deal with the analysis and the mechanism of this phenomenon.²

¹ For the sake of brevity, this sequence of events occurring in the chicken embryo as a result of the several experimental procedures to be described, will be referred to as the "vascular phenomenon."

² In 1928 Sherwood (5) used chicken embryo hearts for experiments on anaphylaxis. The change in frequency and regularity of the heart beat was employed as the test. He succeeded in producing a sensitization of the heart by means of passive transfer of chicken antiserum (rabbit antiserum had no effect).

Method

The following method (Kaufman (6), Szepsenwol (7)) is used to remove the embryos from the eggshell: Fertilized chicken eggs are kept for 66 to 72 hours in the incubator at about 40–42°C. Then the eggshell is cleaned and washed with alcohol. The eggshell is opened, preferably at the small pole, about a third to a half of the shell is carefully removed and the egg poured into a sterile beaker. The beaker is then covered with a sterile watch glass and kept in the incubator at 37–38°C. In the experiments to be cited the sera were added to the chicken embryo drop by drop.

Experiments on Primary Toxicity of Rabbit Type Sera toward the Chicken Embryo

1. *Effectiveness of Active, Inactivated and Reactivated Normal Sera.*—Normal rabbit type sera lose their ability to produce the vascular phenomenon in the chicken embryo after being heated at 56°C. for $\frac{1}{2}$ hour. Hence the first question which arose was whether or not inactivated normal human serum can be reactivated by addition of complement (fresh guinea pig serum). Table I illustrates a typical experiment designed to elucidate this question (Table I).

As is seen, normal human serum inactivated at 56°C. cannot be reactivated in its potency to induce the vascular phenomenon by addition of fresh guinea pig serum.

In order to study the relationship between the destruction of complement and the potency of the phenomenon-producing factor ("factor toxic" to the embryo) in normal rabbit type sera, the following experiment was performed.

Normal human serum was divided into three equal portions. Two of these were placed in a water bath for 20 minutes at a temperature of 49°C. and 51°C. respectively. The complement content of each specimen was then determined as follows:

Decreasing amounts of each of the above heated sera as well as active serum were made up to 0.2 cc. volume with physiological saline. To each of these there was added 0.4 cc. of strongly sensitized sheep cells. The mixtures were placed in the incubator at 37°C. Hemolysis was noted after 30 minutes (Table II).

As is seen from Table II, the complement of human serum was slightly inactivated at 49°C., but completely destroyed at 51°C. These sera were then tested for their effect on the chicken embryo. For this purpose decreasing amounts of the above sera were made up

TABLE I

Effect of Normal Active, Inactivated and Reactivated Human Serum on the Chicken Embryo

Amount of serum	Reaction of the chicken embryo after	
	3 hrs.	8 hrs.
cc.		
0.5 active human	++++	++++
0.25 " "	++	++++
0.15 " "	—	—
0.5 inactivated* human	—	—
0.5 " " + 0.2 cc. fresh guinea pig serum	—	—

— represents no reaction; + to ++++ represent different stages in the development of the phenomenon varying from the beginning contraction of the vascular net (+) to final contraction associated with sinking of the embryo into the depth of the yolk and its death (++++).

* Inactivation in water bath at 56°C. for ½ hour.

TABLE II

Influence of Heat on Complement as Determined by Hemolysis of Sensitized Sheep Cells Produced by Normal Human Serum

Amount of human serum	Extent of hemolysis		
	Active serum	Serum incubated at	
		49°C.	51°C.
cc.			
0.1	C.	C.	0
0.05	C.	M.	0
0.025	M.	M.	0
0.0125	M.	Tr.	0
0.00625	Tr.	Tr.	0
0.003125	Tr.	0	0
0.0	0	0	0

C., complete hemolysis; M., moderate hemolysis; Tr., trace of hemolysis; 0, no hemolysis.

to 1.0 cc. by the addition of physiological saline and then added to the chicken embryo preparation. The results are given in Table III.

Table III shows that at the end of 2 hours serum heated at 49°C.

was less effective against the chicken embryo than the unheated serum. At the end of 8 hours, however, it appeared to be just as potent as the unheated serum. On the other hand, serum heated at 51°C. completely lost its effect. Repeated experiments invariably disclosed this parallelism between the complement content of the serum and its ability to produce the vascular phenomenon in the chicken embryo.

It has been shown (Table I) that the addition of guinea pig serum (complement) to human serum inactivated at 56°C. fails to restore its potency to produce the vascular phenomenon. Since it is known that antibodies of normal serum are relatively thermolabile it seemed of interest to determine whether the addition of complement to serum in-

TABLE III

Effect of Heating on Phenomenon-Producing Potency of Normal Human Serum

Amount of serum	Reaction of chicken embryo after	
	2 hrs.	8 hrs.
cc.		
0.5 active	++++	++++
0.25 "	+++	++++
0.15 "	—	—
0.5 heated at 49°C.	++	++++
0.25 " " 49°C.	+	++++
0.15 " " 49°C.	—	—
0.5 " " 51°C.	—	—

activated at lower temperature could restore this potency. However, on repeated attempts it was found impossible to reactivate human serum which was inactivated at 51°C. even by addition of large quantities of fresh guinea pig serum (0.1 to 0.5 cc.). Nor was such reactivation accomplished by the use of many variations in the method of experiment; *e.g.*, instead of mixing inactivated serum and guinea pig serum before its addition to the chicken embryo, we added first the inactivated serum and several hours later the guinea pig serum.

In this connection it seemed of special interest to study umbilical cord serum inasmuch as this serum is apparently characterized by a poverty of normal antibodies. It was, therefore, surprising to find that two samples of undiluted umbilical cord serum produced a typical vascular phenomenon even though sheep cell hemolysins could not be

demonstrated *in vitro*. The effectiveness of umbilical cord serum which does not contain demonstrable sheep cell hemolysins seems to argue against the identity of the latter with the vascular phenomenon-producing factor. The half diluted umbilical cord serum, however, was without effect. On the other hand, despite demonstrable complement content, such half diluted serum did not reactivate inactivated normal human serum.

2. *The Specific Inhibitory Effect of Guinea Pig Type Sera.*—The vascular phenomenon could be obtained by means of sera of animals belonging to the rabbit type. Up to now, normal sera of man, rabbit,

TABLE IV
Inhibitory Effect of Sera of the Guinea Pig Type on Active Human Serum

Amount of serum				Reaction of chicken embryo after 8 hrs.
cc.		cc.		
0.5 active human	+	0.5 physiological saline		++++
0.5 " rat	+	0.5 " "		++++
0.5 " human	+	0.5 inactivated chicken		—
0.5 " "	+	0.5 " guinea pig		—
0.5 " "	+	0.5 " human		++++
0.5 " "	+	0.5 " rat		++++

rat and pigeon² were found effective, while those of guinea pig,⁴ horse and chicken were ineffective.

Sera of animals of the guinea pig type contain dissolved Forssman antigen. For this reason, it seemed of interest to determine whether sera of the guinea pig type would alter the potency of rabbit type sera in respect to their ability to produce the vascular phenomenon. In order to determine this, the following experiment was carried out.

0.5 cc. of active human serum was mixed with 0.5 cc. of inactivated serum from chicken, rat, guinea pig and man respectively. These mixtures were left at room temperature for 10 minutes and then added to chicken embryos. The reaction resulting after 8 hours is noted in Table IV.

² The pigeon has been described as lacking in complement, and for this reason the mechanism of its effectiveness should be investigated further.

⁴ Guinea pig sera are encountered which contain sheep cell hemolysins; it would be interesting to study the effectiveness of such a serum against the chicken embryo.

As is seen from Table IV, inactivated chicken and guinea pig sera, containing Forssman antigen, inhibit the potency of normal human serum, but inactivated rat and human sera, lacking Forssman antigen, do not display any inhibitory effect.

Experiments on the Toxicity of Forssman Antiserum for the Chicken Embryo

1. *Effectiveness of Inactivated and Reactivated Forssman Antiserum.*—In contrast to normal sera of the rabbit type, inactivated Forssman antiserum can be made effective against the chicken embryo by the addition of fresh guinea pig serum. Each of these is ineffective by itself. One may infer that when the vascular phenomenon is pro-

TABLE V
Effect of Forssman Antisera on the Chicken Embryo

Inactivated sheep cell antiserum		Guinea pig serum	Reaction of chicken embryo after 15 hrs.
Amount	Dilution		
cc.		cc.	
1.0	1:10	0.2	++++
1.0	1:30	0.2	++++
1.0	1:90	0.2	—
1.0	1:10	0.1	—
1.0	1:30	0.1	—
1.0	1:10	0.05	—

duced by the addition of antisera it is necessary for antibody and complement to be present. The following experiment illustrates the quantitative relationship between both factors.

Decreasing amounts of fresh guinea pig serum were made to 0.2 cc. with physiological saline and mixed with 1.0 cc. of various dilutions of an inactivated sheep cell antiserum. Then the mixtures were added to 3 day old chicken embryos and the reaction resulting after 15 hours noted in Table V.

As is seen from Table V, relatively large amounts of fresh guinea pig serum (complement) were necessary in order to reactivate inactivated Forssman antiserum against the chicken embryo. Sometimes smaller amounts (0.1 cc.) of fresh guinea pig serum can reactivate it however. The Forssman antiserum in a dilution up to 1:30 to-

gether with 0.2 cc. fresh guinea pig serum is capable of producing the vascular phenomenon; but not every sheep cell antiserum is effective in so high a dilution. Thus, of 9 sheep cell antisera which reacted well *in vitro*, 4 were ineffective against the chicken embryo even in a dilution of 1:10. In higher concentrations (dilution 1:2 to 1:5) they produced the phenomenon. On the other hand, one sheep cell antiserum was able to induce the vascular phenomenon even in a dilution of 1:200. As a rule antiserum and complement were mixed in a test tube before they were added to the embryo, but there was no difference in the reaction of the embryo after separate addition of both components provided the interval was less than 12 hours.

TABLE VI
Reactivation of Inactivated Forssman Antiserum by Guinea Pig and Chicken Serum

Sheep cell antiserum		Complement	Reaction of chicken embryo after 15 hrs.
Amount	Dilution		
cc.		cc.	++++
1.2	1:10	0.2 guinea pig serum	—
1.2	1:10	0.3 chicken serum	—
1.2	1:10	0.1 " "	—
1.2	1:10	0.05 " "	—
1.2	1:10	0.3 saline	—

The remarkable difference between Forssman antiserum and normal sera of the rabbit type in that the former can be reactivated in its potency toward the chicken embryo by the addition of complement, suggests the possibility of differentiating in this way between normal antibodies of the blood serum and those produced by immunization or diseases.

It is known that bird serum does not complement amboceptors of mammalian sera. Therefore, it seemed of interest to determine whether or not it would be possible for normal chicken serum to be used as complement in the reactivation of inactivated Forssman antisera in their effect on the chicken embryo. For this purpose the following experiment was set up.

Decreasing amounts of chicken serum and guinea pig serum respectively were made up to 0.3 cc. with physiological saline and each mixed with 1.2 cc. of an inactivated sheep cell antiserum in a dilution of 1:10. The mixtures were then added to 3 day old chicken embryos. The results are given in Table VI.

Table VI shows that chicken serum does not restore the inactivated Forssman antiserum to phenomenon-producing potency. Thus, chicken serum does not function as complement in this preparation.

2. *The Specific Inhibitory Influence of Guinea Pig Type Sera on Forssman Antisera.*—Since chicken serum contains dissolved Forssman antigen capable of fixing the corresponding antibodies, we examined the influence of chicken serum on reactivated Forssman antiserum in

TABLE VII
Inhibitory Effect of Chicken Serum on Reactivated Forssman Antiserum

Sheep cell antiserum, dilution 1:2	Guinea pig serum	Saline	Chicken serum	Reaction of the chicken embryo after	
				3 hrs.	12 hrs.
cc.	cc.	cc.	cc.		
0.4	0.2	0.5	0.0	++++	++++
0.4	0.2	0.0	0.5	—	—

order to determine whether an inhibitory effect could be demonstrated. The experiment was carried out in the following way.

To 0.6 cc. of an equal mixture of sheep cell antiserum, fresh guinea pig serum and saline respectively were added (a) 0.5 cc. chicken serum, and (b) 0.5 cc. saline. These mixtures were left at room temperature for 10 minutes and then dropped on 3 day old chicken embryos. The reaction resulting after 3 and 12 hours respectively is noted in Table VII.

As is seen from Table VII chicken serum prevents a reactivated Forssman antiserum from producing the vascular phenomenon.

DISCUSSION

One of the characteristic qualities of the Forssman antigen is its widespread presence in the living world. It is found on the one hand in animals which have no zoological relationship at all, guinea pig,

horse, dog, cat, chicken (animals of the so called guinea pig type). On the other hand, closely related animals may differ in their content of Forssman antigen. Serum of such animals whose organs lack Forssman antigen contains antibodies against this antigen: rabbit, man, cow, duck, hog (animals of the so called rabbit type). In this characteristic the distribution resembles that of the blood group properties of man. In the serum there are always present antibodies against such group qualities as are absent in the individual himself.

The vascular phenomenon of the chicken embryo is produced by Forssman antiserum and by normal sera of the rabbit type. Sera of the guinea pig type are ineffective. Normal rabbit type sera lose their effectiveness after being heated at 51°C . The disappearance of the vascular phenomenon-producing potency parallels the loss of complement. Up to now, it has not been possible to reactivate heat-inactivated normal serum by the addition of complement, in contrast to Forssman antisera which can be easily reactivated by the addition of fresh guinea pig serum. This difference between normal and immune serum discloses a similarity to those antibody functions which play an important rôle in phagocytosis.

The vascular phenomenon as such is to be considered as a sequela of the reaction between the Forssman antigen of the chicken embryo on the one hand and the corresponding antibodies of the added serum on the other. This statement is supported by the following facts.

1. The vascular phenomenon of the chicken embryo is produced only by sera containing Forssman antibodies.

2. Duck embryos, which lack Forssman antigen, do not give the vascular phenomenon.

3. Forssman antisera lose their effectiveness toward the chicken embryo after being absorbed with sheep cells. However, attempts to fix the phenomenon-producing factors of normal sera by absorption with sheep cells, have as yet been inconclusive.

4. Guinea pig type sera containing dissolved Forssman antigen inhibit normal rabbit type sera as well as Forssman antisera in their effectiveness toward the chicken embryo.

These findings parallel the so called inverted anaphylactic shock of guinea pigs, which is characterized by the fact that the antibodies of

Decreasing amounts of chicken serum and guinea pig serum respectively were made up to 0.3 cc. with physiological saline and each mixed with 1.2 cc. of an inactivated sheep cell antiserum in a dilution of 1:10. The mixtures were then added to 3 day old chicken embryos. The results are given in Table VI.

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0.4	0.2	0.0	0.5	—	—

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One of the characteristic qualities of the Forssman antigen is its widespread presence in the living world. It is found on the one hand in animals which have no zoological relationship at all, guinea pig,

horse, dog, cat, chicken (animals of the so called guinea pig type). On the other hand, closely related animals may differ in their content of Forssman antigen. Serum of such animals whose organs lack Forssman antigen contains antibodies against this antigen: rabbit, man, cow, duck, hog (animals of the so called rabbit type). In this characteristic the distribution resembles that of the blood group properties of man. In the serum there are always present antibodies against such group qualities as are absent in the individual himself.

The vascular phenomenon of the chicken embryo is produced by Forssman antiserum and by normal sera of the rabbit type. Sera of the guinea pig type are ineffective. Normal rabbit type sera lose their effectiveness after being heated at 51°C. The disappearance of the vascular phenomenon-producing potency parallels the loss of complement. Up to now, it has not been possible to reactivate heat-inactivated normal serum by the addition of complement, in contrast to Forssman antisera which can be easily reactivated by the addition of fresh guinea pig serum. This difference between normal and immune serum discloses a similarity to those antibody functions which play an important rôle in phagocytosis.

The vascular phenomenon as such is to be considered as a sequela of the reaction between the Forssman antigen of the chicken embryo on the one hand and the corresponding antibodies of the added serum on the other. This statement is supported by the following facts.

1. The vascular phenomenon of the chicken embryo is produced only by sera containing Forssman antibodies.

2. Duck embryos, which lack Forssman antigen, do not give the vascular phenomenon.

3. Forssman antisera lose their effectiveness toward the chicken embryo after being absorbed with sheep cells. However, attempts to fix the phenomenon-producing factors of normal sera by absorption with sheep cells, have as yet been inconclusive.

4. Guinea pig type sera containing dissolved Forssman antigen inhibit normal rabbit type sera as well as Forssman antisera in their effectiveness toward the chicken embryo.

These findings parallel the so called inverted anaphylactic shock of guinea pigs, which is characterized by the fact that the antibodies of

the serum are the actuating factor, while the antigen is present in the cells and tissues of the reacting organism itself.⁵

The significance of complement for anaphylactic reactions has been discussed for many years. Dale's (8) demonstration of anaphylactic reactions on the isolated uterus suggests that complement is unnecessary for anaphylaxis. Hyde (9), however, failed to induce the inverted anaphylactic shock in guinea pigs which did not possess complement in their blood. On the other hand, Doerr and Pick (10) were able to produce a typical anaphylactic shock in the adult chicken by the intravenous injection of an inactivated Forssman antiserum without addition of complement, an observation which we can confirm. However, for the production of the vascular phenomenon with the chicken embryo, complement is absolutely necessary, as has been shown above. The necessity of adding complement to inactivated Forssman antiserum suggests that either the embryo itself does not contain sufficient complement or that its complement will not function with mammalian antibodies.

It is to be borne in mind that the Forssman antigen is not a uniform substance, but consists of various components with different corresponding antibodies. This fact may perhaps help to explain the observation that Forssman antisera are encountered with sheep cell hemolysin titer 1000 times higher than that of a normal human serum, yet with a potency to produce the vascular phenomenon that may be almost the same as that of the normal serum. In this connection it may be mentioned that umbilical cord serum produced the vascular phenomenon in the chicken embryo, in spite of the fact that demonstrable sheep cell antibodies were not present. As yet it is impossible to state whether the difference between the Forssman antibodies of the agglutinin and hemolysin type and those responsible for the production of the vascular phenomenon are due to differences in the titer of partial antibodies or to qualitatively different antibody functions.

⁵ Regarding nature and mechanism of the inverted anaphylactic shock, cf. Doerr, R., in Bethe, A., von Bergmann, G., Embden, G., and Ellinger, A., *Handbuch der normalen und pathologischen Physiologie*, Berlin, Julius Springer, 1929, 13, 757. Forssman, J., in Kolle, W., and von Wasserman, A., *Handbuch der pathogenen Mikroorganismen*, Jena, Gustav Fischer, 3rd edition, (Kolle, W., Kraus, R., and Uhlenhuth, P.), 1928, 3, Liefg. 23, 469.

SUMMARY

1. The 3 day old chicken embryo removed from its shell is a suitable test object for the demonstration of primary serum toxicity. Addition of normal rabbit type sera as well as Forssman antiserum causes the vascular network to contract and the embryo sinks in the yolk and dies.

2. Only sera of animals of the so called rabbit type produce this phenomenon. Sera of the guinea pig type are ineffective.

3. Heating to 51°C. destroys the complement content of normal human serum as also its effectiveness to produce the vascular phenomenon.

4. Up to the present it has not been possible to reactivate heat-inactivated normal serum by the addition of complement, while inactivated Forssman antiserum can be easily reactivated.

5. The vascular phenomenon of the chicken embryo is produced not only by the addition of a mixture of Forssman antiserum and complement but also by separate addition of both components.

6. Guinea pig type sera, containing dissolved Forssman antigen, are not only ineffective but actually exert an inhibitory influence on effective rabbit type sera as well as on Forssman antiserum.

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CULTIVATION OF THE VIRUS OF GRASSERIE IN SILK- WORM TISSUE CULTURES

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PLATES 21 AND 22

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Adequate evidence has been presented by Glaser and Chapman, 1913, Paillot, 1924, Glaser, 1927, and others (Glaser in Rivers, 1928) to show that the polyhedral diseases of caterpillars are due to specific filterable viruses. Since many filterable viruses of vertebrates have been propagated in tissue cultures (Carrel in Rivers, 1928; also, vaccinia (Li and Rivers, 1930, Rivers and Ward, 1933); vesicular stomatitis (Carrel, Olitsky, and Long, 1928); fowl pox (Findlay, 1928); Virus III (Andrewes, 1929, *a*, *b*); herpes (Andrewes, 1930); foot-and-mouth disease (Hecke, 1930, Maitland and Maitland, 1931); common cold (Dochez, Mills, and Kneeland, 1931); pseudorabies (Traub, 1933); yellow fever (Haagen, 1934), as well as two plant viruses in growing excised root tips (White, 1934)), it seemed worth determining whether a virus disease of an invertebrate could be similarly maintained, and whether it would behave in culture in a manner like that of virus diseases of vertebrates. Moreover, cultures of a polyhedral virus might be expected to be of special value in studies on the nature of inclusion bodies. It is well known that many filterable viruses bring about the formation, within certain cells of the host organism, of nuclear or cytoplasmic inclusion bodies, all of highly characteristic appearance, but none so definite and easily observed without fixation or staining as the polyhedral nuclear inclusions which occur in silkworms affected by grasserie.

The culture of silkworm tissue, the propagation in these cultures of the virus of grasserie, and some observations on the formation of

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polyhedra in cells living *in vitro* will all be detailed in the succeeding portions of this paper.

General Methods

A few general remarks must first be made. The silkworms were raised from healthy stock obtained from Dr. Glaser and maintained free of virus for several years. They were kept in a room carefully guarded from the introduction of infective material. During the entire breeding season no cases of grasserie occurred among the stock of silkworms. Indeed, very few stock silkworms were sick from any cause whatever, except during the late fall, when the food was of inferior quality and a number of deaths from bacterial disease occurred. Experimental silkworms were kept singly in half pint bottles and were generally treated in the manner described by Glaser and Lacaille (1934). Material which was to be tested for infectivity was diluted with water to a known extent and 0.01 to 0.02 cc. of it fed to caterpillars previously starved for 1 day, using usually 5 caterpillars for each dilution. In this way rough titrations of the virus could be carried out.

Cultivation of Silkworm Tissue

All reported attempts at the cultivation *in vitro* of insect tissue have met with relatively slight success.

Goldschmidt (1915) obtained growths of the follicle cells of male gonads removed from pupae of *Samia cecropia*. These growths occurred in hanging drops of hemolymph. No mitoses could be found. Glaser (1917) observed the formation of syncytia by the amebocytes in hanging drops of silkworm blood. Lazarenko (1925) saw similar amebocyte syncytia in hanging drops of blood of the larva of the beetle, *Oryctes nasicornis*, and the writer has noted such syncytia in hanging drops of blood of the cockroach, *Periplaneta americana*, of silkworm larvae, and of pupae of *Samia cecropia*. Lazarenko found that the syncytia were formed by an extreme flattening, spreading out, and anastomosing of the amebocytes, and that no multiplication of these cells occurred. Our own observations support this view. Frew (1928), using the imaginal discs of blow-fly larvae in larval or pupal body fluid sterilized by Berkefeld filtration, was unable to get multiplication of the cells. However, entire imaginal discs of the legs, when placed in pupal body fluid, evaginated and underwent a partial development into segmented limbs. Lewis and Robertson (1916) developed a balanced salt solution containing glucose and grasshopper bouillon or peptone, in which the testis cells of *Chorthippus curtipennis* lived for some time and underwent normal mitotic division. Belař (1929) used a similar modified Ringer's solution, but without peptone or bouillon, to observe mitosis in spermatocytes of the grasshopper, *Chorthippus lineatus*. Neither of the latter two workers noted division in any cells other than the germ cells.

In the present investigation the effects were tried of a number of balanced salt solutions on the tissues of full grown larvae of *Bombyx mori*. All the solutions had a concentration approximately equivalent to a 0.05 M NaCl solution, as this was at the center of the favorable osmotic pressure range, and a pH of 6.7, since this is the pH of silk-worm hemolymph (Glaser, 1925, Demyanovskii *et al.*, 1932). A medium was finally found in which good initial cultures could be

TABLE I
Composition of the Culture Media

	Solution A		Solution B	
	cc. per 100 cc.	moles per liter	cc. per 100 cc.	moles per liter
Maltose, 0.3 M.....	20.0	0.06	20.0	0.06
NaCl, 0.3 M.....	5.0	0.015	0.4	0.0013
MgCl ₂ ·6H ₂ O, 0.2 M.....	0.5	0.001	0.5	0.001
CaCl ₂ , 0.2 M.....	0.5	0.001	0.5	0.001
NaH ₂ PO ₄ ·H ₂ O, 0.2 M.....	0.75	0.0015	0.75	0.0015
K ₂ HPO ₄ , 0.2 M.....	0.75	0.0015	0.75	0.0015
Egg albumin digest, 0.137 M*.....	0	0	10.0	0.0137
Distilled water.....	72.5		67.1	
Freezing point depression, °C.†.....	0.285		0.290	
Nitrogen per cc., mg.†.....	0		1.19	

The media were prepared by mixing the indicated amounts of sterile 0.3 or 0.2 M solutions with sterile distilled water. The stock solutions were sterilized by autoclaving, except the maltose and the digest, which were filtered through a Berkefeld N.

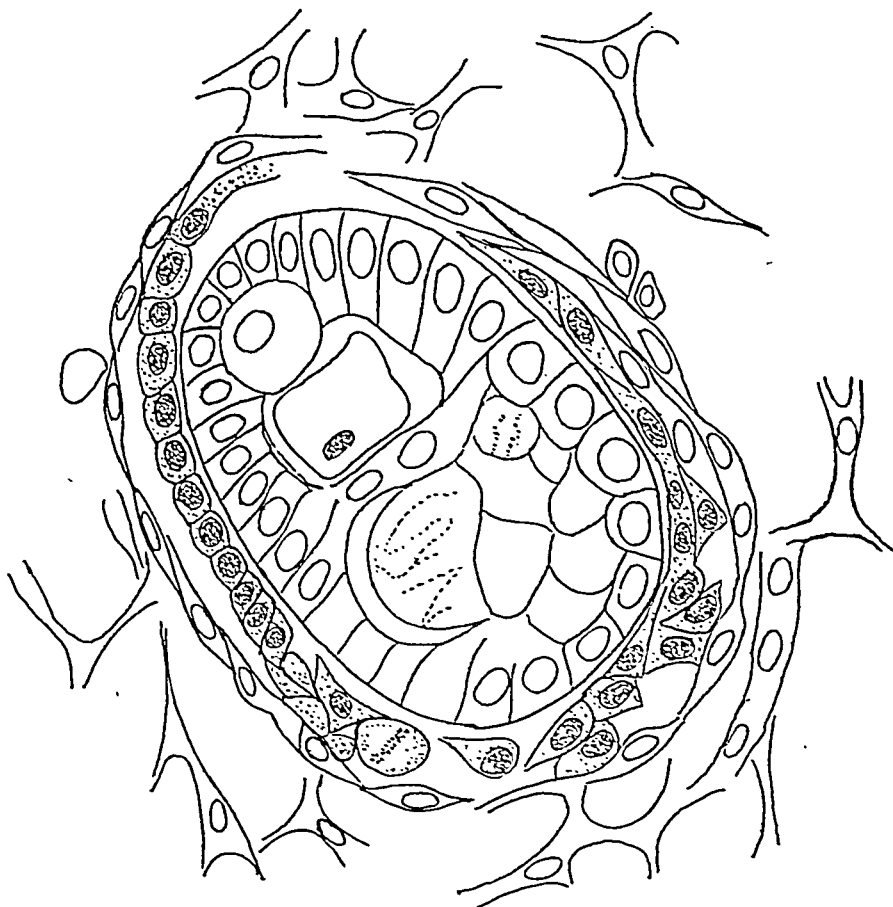
* The molarity of the digest was calculated from the amount of NaOH needed to neutralize it and from its final volume after heating. That the figure obtained is sufficiently accurate is shown by the fact that the freezing point depression of Solution B is only slightly higher than that of A.

† These determinations were made by Dr. William F. Bruce.

obtained of a certain silkworm tissue derived from the female gonads. The compositions of this medium (A) and of a modification of it (B) are given in Table I.

Note that the media contain maltose. Neither sucrose nor glucose in equimolecular amounts gave growths as good as those obtained with maltose. Medium B differs from A in that it contains an egg albumin digest prepared in the manner of Baker (1929). Growth was usually greater in Medium B than in A.

especially if the media were used without the addition of silkworm serum. The best growths were obtained when the medium contained from 5 to 25 per cent (usually 10 per cent) silkworm serum. The latter was secured by bleeding several large silkworms aseptically from the proleg, pooling the blood, and centrifuging out the blood cells.

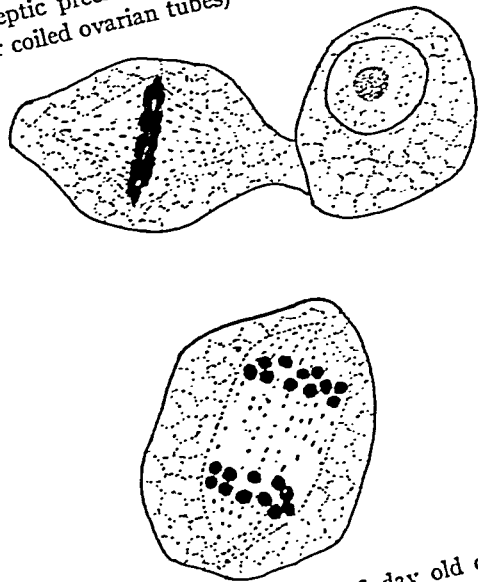


TEXT-FIG. 1. Section through ovarian tube of 5th instar silkworm. The stippled cells represent the layer which grew in culture.

The tissue cultures were prepared in the following manner. Full grown female silkworms were immersed in 1:1000 mercuric chloride solution for 30 to 45 minutes. This treatment did not injure the caterpillars as, if they were subsequently washed and allowed to dry out, they were completely harmless and could be handled. After immersion in mercuric chloride solution, the caterpillar was washed in sterile water and was then bled into a Petri dish. It was next

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placed in another small Petri, and with fine sharp scissors the entire dorsal wall of the 8th segment was cut off (care being taken not to cut into the alimentary canal) and placed in the blood previously obtained. Usually the imaginal buds of the ovaries came out along with the dorsal body wall of the 8th segment. Sometimes they did not, and then it was necessary to dissect them out with glass needles and place them in the dish with the blood. In the former case the gonads were dissected away from the body wall, also with glass needles. All the instruments used were sterile, and aseptic precautions were observed throughout. Each gonad (which contains four coiled ovarian tubes) was divided into 3 to 4 pieces. Each

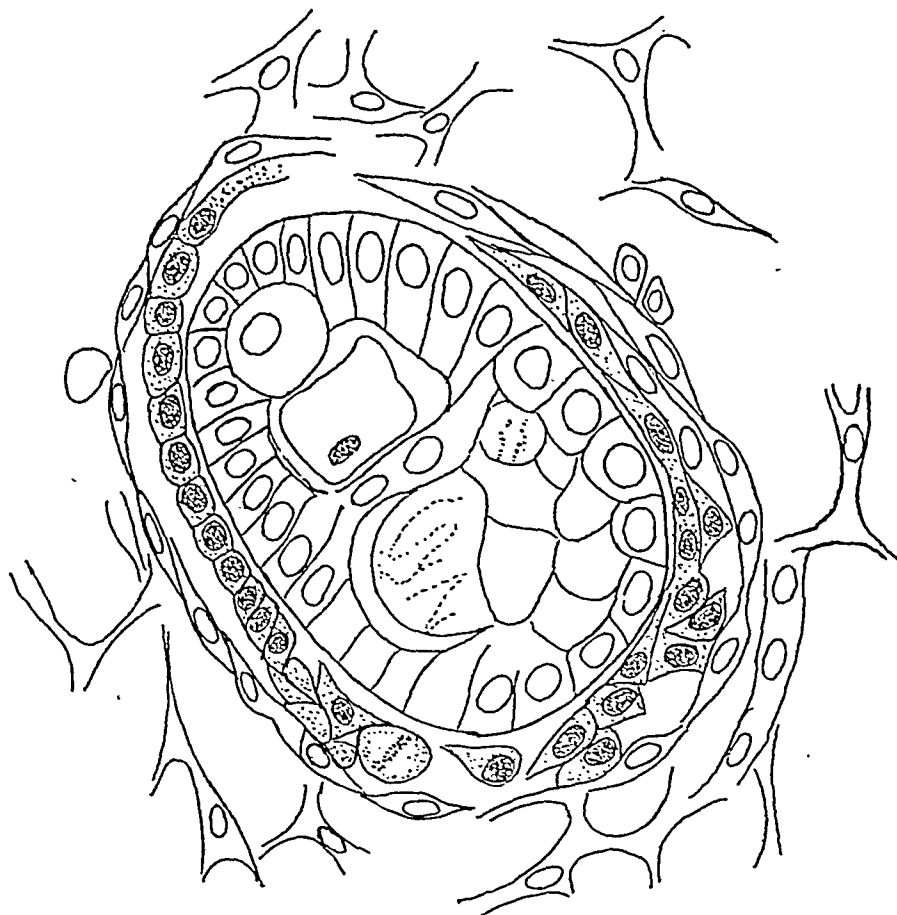


TEXT-FIG. 2. Mitotic figures from a 3 day old culture stained with crystal violet.

fragment of tissue so obtained (about 0.5 to 1 mm. in diameter) was set up in a hanging drop (0.005 cc.) of culture fluid on a sterile cover-slip vased on to a microculture slide. The cultures were kept in an incubator at 27.5–28.5°C. By these methods very few cultures were ever contaminated by bacteria. Nevertheless, rarely would more than 75 per cent of the cultures prepared at one time be successful. Sometimes the lack of success of a culture could be attributed to an insufficient number of cells of the right type (see below) in the explant, but for other cases there was no obvious explanation. It was noted that tissues from caterpillars with colorless blood generally gave better growths than those from caterpillars with yellow blood.

In successful cultures small numbers of wandering connective-tissue-like cells appear after 1 or 2 days incubation. These cells come

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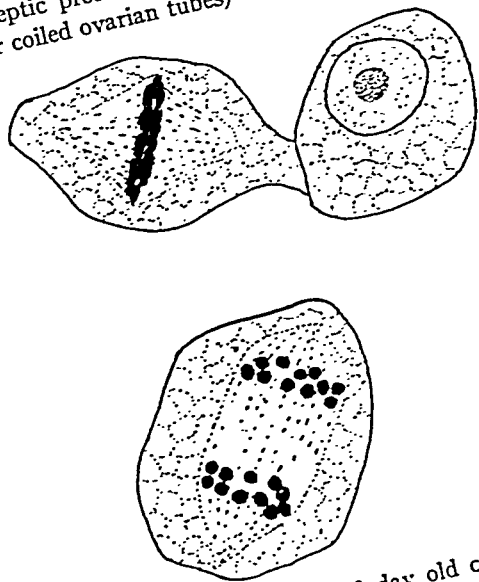


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placed in another small Petri, and with fine sharp scissors the entire dorsal wall of the 8th segment was cut off (care being taken not to cut into the alimentary canal) and placed in the blood previously obtained. Usually the imaginal buds of the ovaries came out along with the dorsal body wall of the 8th segment. Sometimes they did not, and then it was necessary to dissect them out with glass needles and place them in the dish with the blood. In the former case the gonads were dissected away from the body wall, also with glass needles. All the instruments used were sterile, and aseptic precautions were observed throughout. Each gonad (which contains four coiled ovarian tubes) was divided into 3 to 4 pieces. Each



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In successful cultures small numbers of wandering connective-tissue-like cells appear after 1 or 2 days incubation. These cells come

from the tissue forming the lining of the ovarian tubes (Text-fig. 1). The cells forming the loose stroma of the larval gonad (Machida, 1926) do not grow in culture. By the 3rd day, the lining cells are very numerous, and they become more so on the 4th and 5th days. Usually the cells then remain in good condition for another week or two. They have numerous fine pseudopodia, and exhibit slow ameboid movement. The growth takes place in 2 layers, one against the cover-slip and one on the surface of the hanging drop. Fig. 1 is a photograph of a small portion of a 6 day old growth, with only the layer on the cover-slip in focus. Several cultures which were fixed and stained showed mitotic figures (Text-fig. 2). Cells growing in media containing silkworm serum have more cytoplasmic granules than those in serum-free media.

The cultures could not be kept going indefinitely, although a few first subcultures were successful. The initial cultures were, however, perfectly suitable for the cultivation of the polyhedral virus.

No tissue cultures were obtained when pieces of silkworm nerve, muscle, fat, silk-gland, leg or wing bud, or male gonad were explanted under the conditions suitable for growth of the lining cells of the ovarian tubes.

Cultivation of the Virus

Initial cultures of the silkworm polyhedral virus have been repeatedly obtained simply by explanting the gonad tissue into culture medium containing the virus. One example will suffice.

Blood from a typical case of grasserie was drawn aseptically and diluted 1 in 25 with Medium A. The mixture was centrifuged and the clear supernatant liquid stored 3 days in the ice box while its sterility was being tested in a broth tube inoculated from it. 1 part of this liquid was then further diluted with 3 parts of Solution B to give a total virus concentration of 1 in 100. Another part of the liquid was diluted with 3 parts of a mixture of Solution B and silkworm serum, giving a virus concentration of 1 in 100 and a serum concentration of 25 per cent. Proper control mixtures were made of Solutions A and B, and A and B with serum. Each of the 4 mixtures was used as the medium for 3 hanging drop cultures of female gonad tissue. The drops, in this as in all the other experiments, were measured out with a fine pipette to be 0.005 cc. in volume.

After 1 day of incubation, all the cultures were about the same and showed a few outwandering cells. On the 2nd day, growth had begun in all but one control

culture, but there was no growth in cultures containing the virus. On the 3rd day, intranuclear polyhedra began to appear in some of the ovarian tube lining cells in cultures infected with virus both with and without serum. Subsequently, all but one of the controls gave typical excellent cultures. In the infected cultures more polyhedra appeared, and in those containing serum a fatty degeneration of the cells soon set in.

On the day when the cultures were set up, a portion of the original virus mixture was diluted with water to give virus concentrations of 1:10,000, 1:100,000, and 1:1,000,000, and each dilution was fed to 5 silkworms. None of the 15 worms thus fed contracted the disease. After 4 days' incubation, an infected culture in the medium with serum was teased up and diluted with water to give virus concentrations (in terms of the original virus) of 1:100,000, 1:1,000,000, and 1:5,000,000. Several of the worms fed the first two dilutions came down with grasserie, showing that the virus had multiplied more than 1000 times. After 9 days' incubation an infected culture in the medium without serum was similarly diluted to give virus concentrations of 1:1,000,000 and 1:10,000,000. 2 of the 5 worms fed the first, and 1 of the 5 fed the second dilution contracted typical polyhedral infections, showing that in this culture the virus had multiplied over 10,000 times.

The strain of virus thus obtained in culture was subcultured by transferring a bit of infected tissue to a healthy tissue culture, in which a large outgrowth of cells had already occurred. The strain, started July 28th, was subcultured 9 times, the last subculture being performed October 19th. It regularly brought about the formation of typical polyhedra, usually within 2 days after inoculation. Its activity was further tested by feeding 3rd, 4th, and 5th passage cultures (diluted with water) to silkworms, and it was found to be capable of producing the disease.

In this strain the subcultures were not made in a quantitative way, so that no estimate of the extent of multiplication is possible. Another strain, however, was subcultured in the following manner.

After the polyhedra were well formed in any given culture, and the cells were beginning to die (usually 1 week after inoculation, although longer intervals were occasionally permitted between subcultures) the culture was teased up in 0.25 cc. of Solution A or B. 0.005 cc. of this dilution was then added to a healthy culture from 3 to 10 days old, usually 4, effecting a further dilution of 1 in 2, so that at each subculture the virus was diluted 1:50 except in the 1st subculture when it was diluted 1:100. This strain of virus was carried through 8 passages (7 subcultures) from Aug. 27th to Oct. 26th. The 2nd and 7th passage cultures were each stored 2 weeks in the refrigerator before being subcultured. In the 7th subculture the concentration of the original virus was 1:156,250,000,000,000, yet polyhedral bodies formed in the usual manner.

When this strain was started the virus used was titrated in silkworms at 1:1000, 1:10,000, and 1:100,000. None of these dilutions produced the disease. Third passage culture virus was titrated in silkworms and gave two cases at a dilution of 1:1,250,000,000 of the original virus (the highest dilution used). There can thus be no doubt that the polyhedral virus of silkworms can multiply and form polyhedral bodies in ovarian tube lining cells living *in vitro*.

In an attempt to discover whether the virus could multiply in other silkworm tissues, the following experiment was performed.

One of the 3rd passage cultures (the same as used for the worm titration above) of the strain last described was diluted in the usual way with 0.25 cc. of culture medium. 0.005 cc. was then added to each of two 4 day old ovarian tube lining cell cultures and also to similar cultures containing explants of fat, silk-gland, and muscle tissue, also 4 days old. In the ovarian tube lining cell cultures, polyhedra appeared on the 2nd day.

After 6 days' incubation, 1 of each of the 4 types of cultures was titrated in silkworms, using, for the lining cell cultures, dilutions of 1:6,250,000,000 and of 1:62,500,000,000, for the fat, silk-gland, and muscle cultures only a dilution of 1:6,250,000,000. The infected lining cell cultures, even at the higher dilution, produced the disease in caterpillars, but no cases appeared among the worms fed from cultures of the other three tissues. That the virus did not multiply in silk-gland and muscle tissue is perhaps not surprising, since polyhedra do not form in these tissue *in vivo*. But in the fat-body polyhedra do form *in vivo*. That they could not be detected and that the virus did not multiply in fat-body *in vitro* may be due to the fact that the fat cells do not grow as do the lining cells, and indeed may survive only a short time. In this connection the following observations of Andrewes (1929, *b*) are of interest. He found that while Virus III multiplied and formed inclusions in cultures of rabbit testis, no inclusions were formed in cultures of liver, spleen, kidney, or bone marrow, even though Virus III *in vivo* can infect many different tissues. These latter tissues, however, did not survive as well under the culture conditions as did the testis tissue.

Two strains of polyhedral virus were started with blood from infected silkworms diluted with culture medium and passed through a

Berkefeld N filter. These were not kept going. Subcultures of the other strains had to be discontinued in the late fall when silkworms were no longer available.

Observations on the Formation of Polyhedra in Tissue Culture

Although cytoplasmic or nuclear inclusion bodies are characteristic of many filterable virus diseases (Cowdry in Rivers, 1928), relatively few investigators have concerned themselves with the behavior of these bodies in tissue cultures of viruses. Rivers, Haagen, and Muckenfuss (1929) first reported the appearance of Guarnieri bodies in cultures of adult rabbit cornea infected with vaccinia. At the same time Andrewes (1929, *a, b*) described the formation of typical intranuclear inclusions in the interstitial cells of rabbit testis cultures infected with Virus III, and also in tissue cultures of herpes virus (1930). Haagen (1934) observed nuclear inclusions in rabbit cornea and testis cultures infected with yellow fever virus, while Traub (1933) found the inclusions characteristic of pseudorabies virus to occur in only about 60 per cent of cultures in which the virus had multiplied, as judged from titration experiments.

In the writer's work with silkworm grasserie virus, typical intranuclear polyhedra were always found in at least some of the cells of an infected tissue culture. The number of cells showing well formed polyhedra varied, not with the source of virus used for infection, but with the condition of the tissue culture at the time of infection and during the next few days. Cultures in which the outgoing cells were healthy at the time of infection, as judged from their activity and the appearance of the nucleus and cytoplasm, gave the best and most rapid polyhedral body formation. In such cultures, polyhedra frequently begin to appear within 24 hours after infection, and are present in most of the cells within 48 hours. During several succeeding days the polyhedra increase in size and number. Most of the infected cells meanwhile remain superficially in good condition; *i.e.* the cytoplasm looks like that of uninfected cells and the cells show amoeboid movement. Fig. 2 is a photograph, taken 5 days after infection, of a 4th subculture of the strain of virus first discussed in the preceding section. Compare this with Fig. 1, the photograph of an uninfected tissue culture. Note that the infected cells have hypertrophied and

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have wandered out into the medium. Thus it appears that more and larger polyhedral bodies are formed in active healthy cells than in less active, less healthy cells. This may be taken to indicate that polyhedra are a product of cell activity under the influence of the virus. It is interesting to note that Andrewes (1929, *b*) observed that the inclusions of Virus III were subsequently formed if the tissue cultures were incubated for 48 hours or less before addition of the virus, but they were not formed if the tissues were incubated for 72 hours or more before addition of the virus. In the latter case the cells were probably in poor condition.

Individual cells of infected cultures, at the early stages, were kept under observation for varying periods of time, and all changes in their appearance recorded by means of sketches. Some of these drawings are given in Text-fig. 3. They show that the infected cells changed in shape, that new polyhedra appeared, and that the existing polyhedra increased in size. Within a period of 3 hours a new polyhedral body could become large enough to be visible with the magnification of $\times 1800$. Note in the drawings of Text-fig. 3 that the larger inclusion bodies are of definitely polyhedral shape even when they are not crowded together. Hence the polyhedral form of the bodies cannot be the result of mechanical pressure, as happens when many bodies ordinarily spherical are crowded together in a confined space.

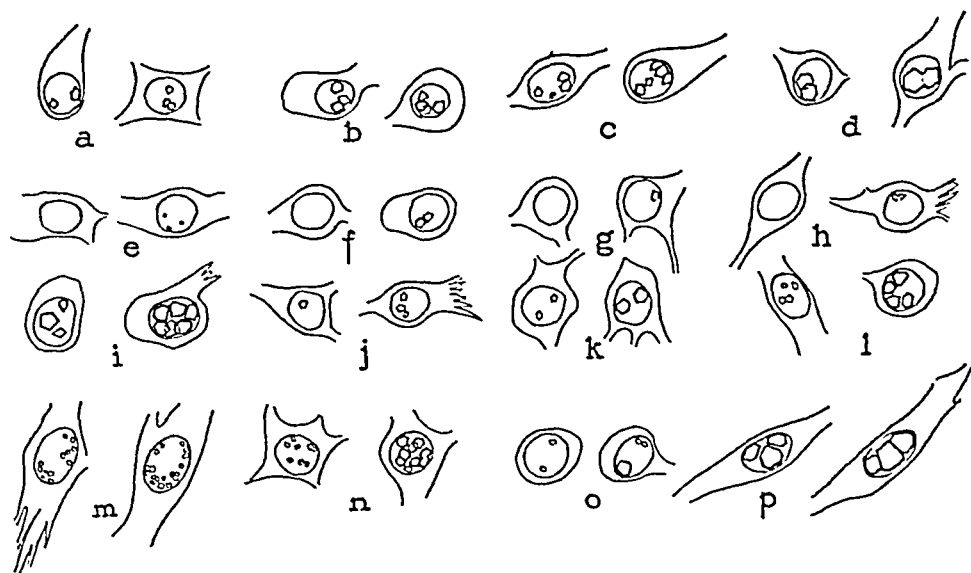
DISCUSSION

The virus of silkworm grasserie apparently behaves in culture much as do the viruses of vertebrates. It multiplies only in the presence of active living cells, it brings about the formation of typical inclusion bodies, and it does not, with successive tissue culture passage, lose any of its characteristic properties.

One other point remains to be discussed. The ovarian tube lining cells of silkworms multiply extensively during the pupal stage to keep pace with the growth of the ovarian tubes, and eventually they form a tissue of the adult moth. Now adults of *Bombyx mori* are completely refractory to the polyhedral disease. Even older pupae, in which the adult tissues are already formed cannot be infected with the virus, although very young pupae are susceptible (Glaser in

that practically all contain polyhedra. The number and size of the polyhedra in individual cells, however, vary enormously.

About a week after infection of a culture, the cells begin to die; *i.e.*, the cytoplasm becomes dense and granular and movement ceases. Some of the dead cells burst and liberate the contained polyhedra. In some cultures, this process continues until the culture has degenerated into a mass of tissue debris and large numbers of free polyhedra (Fig. 3). In other cultures most of the polyhedra are retained within the dead cells.



TEXT-FIG. 3. Stages in the formation of polyhedra in tissue culture. Each pair of drawings represents the same cell at intervals of: for *a* to *c*, 3 hours; for *f* to *h*, 8 hours; for *i* to *p*, 12 hours.

When tissue cultures which contained degenerating cells were infected with polyhedral virus, the appearance of the first polyhedra was delayed, and might take as long as 4 days. No polyhedra ever formed in cells already degenerate, while the polyhedra that formed in other wandering cells of such cultures remained small and few in number. In some very poor cultures, in which the outgoing cells were few and already degenerate at the time of inoculation, polyhedra appeared only in some of the ovarian tube lining cells which had remained within the explant. Such cells might be expected to have a better chance of survival under adverse conditions than cells which

have wandered out into the medium. Thus it appears that more and larger polyhedral bodies are formed in active healthy cells than in less active, less healthy cells. This may be taken to indicate that polyhedra are a product of cell activity under the influence of the virus. It is interesting to note that Andrewes (1929, *b*) observed that the inclusions of Virus III were subsequently formed if the tissue cultures were incubated for 48 hours or less before addition of the virus, but they were not formed if the tissues were incubated for 72 hours or more before addition of the virus. In the latter case the cells were probably in poor condition.

Individual cells of infected cultures, at the early stages, were kept under observation for varying periods of time, and all changes in their appearance recorded by means of sketches. Some of these drawings are given in Text-fig. 3. They show that the infected cells changed in shape, that new polyhedra appeared, and that the existing polyhedra increased in size. Within a period of 3 hours a new polyhedral body could become large enough to be visible with the magnification of $\times 1800$. Note in the drawings of Text-fig. 3 that the larger inclusion bodies are of definitely polyhedral shape even when they are not crowded together. Hence the polyhedral form of the bodies cannot be the result of mechanical pressure, as happens when many bodies ordinarily spherical are crowded together in a confined space.

DISCUSSION

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Rivers, 1928). Since at least one tissue of the adult has now been shown to be susceptible when obtained before the adult is formed and grown *in vitro*, one may suppose tentatively that the immunity of the adult is a humoral and not a tissue immunity.

SUMMARY

A medium has been developed in which certain cells from the gonads of female silkworms multiply and live for periods of 2 to 3 weeks.

In such tissue cultures, strains of silkworm grasserie virus were maintained in successive passages up to the number of ten. The virus multiplied greatly and typical polyhedral bodies formed in the cells of infected cultures.

I take pleasure in acknowledging the help and advice of Dr. R. W. Glaser.

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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Photograph of normal 6 day old culture. $\times 445$.

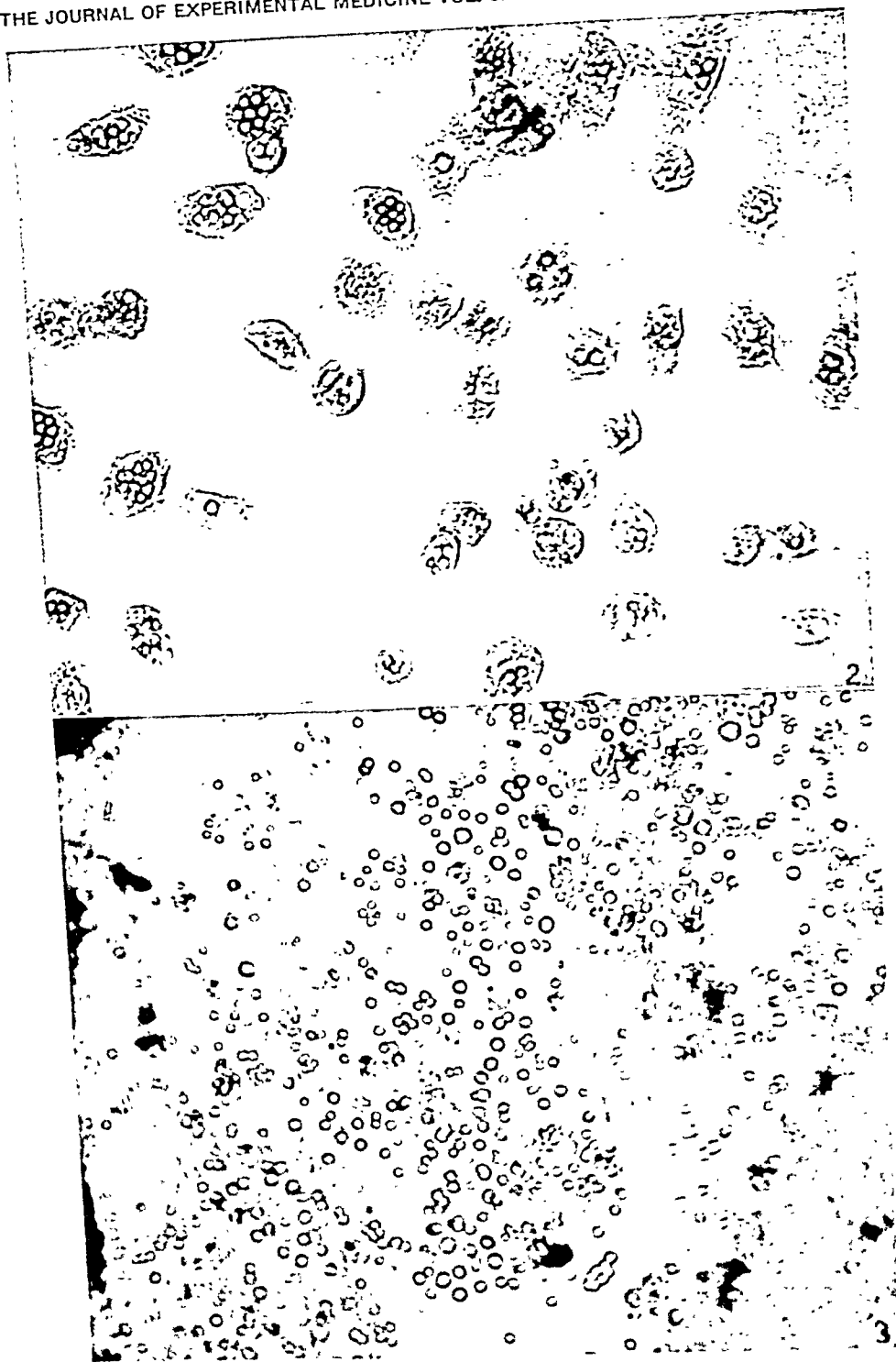
PLATE 22

FIG. 2. A culture 5 days after infection with polyhedral virus. $\times 445$.

FIG. 3. A culture 8 days after infection with polyhedral virus. $\times 445$.



Fig. 1. (A) J. A. G. 1954



STUDIES ON THE HEMOLYTIC STREPTOCOCCUS OF HUMAN ORIGIN

I. OBSERVATIONS ON THE VIRULENT, ATTENUATED, AND AVIRULENT VARIANTS

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PLATE 23

(Received for publication, January 11, 1935)

It could be said with little fear of contradiction that the hemolytic streptococcus presents a more hazy picture to bacteriologists and immunologists than does an organism like the pneumococcus. Its study presents many difficulties. In the first place, many strains which are pathogenic for man may show little or no primary virulence for animals. In the second place, antisera cannot be produced readily and consistently against all pathogenic strains. In the third place, although it is certain that many different types exist, typing by agglutination is full of pitfalls, both in technique and in the interpretation of results.

It seemed to us that the foundation for any work on the hemolytic streptococcus was a careful study of the characteristics of the organism on first isolation from the body, and the ways in which it changed on mouse passage or culture passage; and the results of this part of the work are recorded in this first paper. We were aided in this investigation by the studies of several workers in this field, although it was sometimes difficult to correlate their descriptions of the variants of the hemolytic streptococcus with our own observations.

Cowan (1) was the first to correlate cultural characteristics with virulence. She described a virulent S colony and an avirulent R colony. These variants were apparently found in stock laboratory cultures.

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Todd (2), working with freshly isolated strains from cases of puerperal septicaemia, noted three variants. He found that the organism on primary subculture was virulent for mice, and developed into matt surfaced colonies on chocolate human blood agar plates. After serial cultivation on blood agar, the virulence was diminished, but the colony form remained unchanged. The virulence could be restored by mouse passage or cultivation in human serum. The variant characterized by decreased virulence was designated the matt attenuated form. Complete and permanent loss of virulence resulted from prolonged subcultivation of the matt attenuated form. The colony of this avirulent variant had a glossy surface.

Loewenthal (3) isolated a strain of hemolytic streptococcus from a mouse epidemic. From the original form isolated, he derived three additional variants. The organism obtained directly from the epidemic was mouse-virulent, produced small, compact, conical colonies which grew flocculently in serum broth, and was designated the N (*Nadelkopf*) variant. The N form, on subsequent mouse passage, threw off two variants: the M (mucous) and the O (*ohne Typspezificität*). The M was mouse-virulent, had a slimy colony, and grew diffusely in serum broth. The O was mouse-virulent, and resembled the pneumococcus in its cultural and antigenic characteristics. It lacked type specificity. The R, or fourth, variant was obtained by prolonged cultivation and was distinguished by its avirulence for mice and a cultural similarity to *Streptococcus viridans*.

Dawson and Olmstead (4), in studying strains of recent human origin, found three variants. The colonies of these three variants on neopeptone rabbit blood agar plates were (1) mucoid, (2) smooth convex, (3) smooth flat. They confirmed Loewenthal's observation that the mouse passage of a non-mucoid variant frequently changed it into the mucoid form. It was also noted that the mucoid form was more virulent for mice than the non-mucoid forms, and they thought that human infections from which they isolated the mucoid type were more severe than those caused by the smooth or non-mucoid variants.

In our own work on the variants of the hemolytic streptococcus, we have laid the chief emphasis on the virulent variants and the characteristics which distinguish them from the avirulent forms, in the hope that we would gain a better insight into streptococcal infection, but we have attempted to make the study as comprehensive as possible in the time available.

The details of the experimental methods and results are as follows:

Experimental Methods

(a) *Source of Material*.—The majority of the organisms were obtained from blood cultures and direct platings of pus from human lesions. A few strains were isolated from normal throats. The strains were preserved by picking a colony

into a meat tube, incubating for 24 hours, covering the culture with mineral oil, and storing in the refrigerator.

In every case, whether blood culture or laboratory culture, the strain was plated out and carefully studied to determine whether the culture contained one or more variants.

We are indebted to Dr. Lancefield of The Rockefeller Institute, who kindly sent the matt virulent, matt attenuated, and glossy avirulent variants of three strains with which she and Dr. Todd (5) worked some years ago. To Dr. Loewenthal we owe an N and an O variant.

(b) *Cultivation.*—

1. *Blood Agar Plates.*—These have the following composition:

Saline.....	100.0 cc.
Neopeptone (Difco).....	2.0 gm.
NaOH N/1.....	0.5 cc.
Glycerine.....	1.0 cc.
Agar.....	1.5 gm.
Horse blood.....	15.0 cc.

The first five ingredients are sterilized at 10 pounds for 15 minutes, cooled, the horse blood added, and the plates poured. These plates are soft, but if more agar is added the colony forms of some of the variants are not characteristic. This medium dries out quickly, even in the ice box, and must be used immediately after pouring. The plates are incubated at 37°C. for 15 hours. If a longer incubation is more convenient, a 30°C. incubator should be used. The colonies are examined by reflected light under the colony microscope.

2. *Serum Neopeptone Water.*—The neopeptone water has the following composition:

Distilled water.....	100.0 cc.
Neopeptone (Difco).....	2.5 gm.
Dextrose.....	0.05 gm.

The mixture is boiled, adjusted to pH 7.3, passed through a paper filter, tubed in appropriate amounts, and autoclaved at 10 pounds for 15 minutes. For primary cultivation from blood cultures or meat tubes, 5 per cent horse serum is added to the neopeptone water. The characteristics of the growth (diffuse or flocculent) are observed after overnight incubation at 37°C. Secondary cultures for use in phagocytosis are made by adding 1 drop from the primary culture to 4.0 cc. of a mixture of equal parts of neopeptone water and horse serum. These are grown at 37°C. until the first obvious turbidity appears. This usually occurs after about 2 to 3 hours. Such cultures may be used immediately for phagocytosis, or may be stored on ice for as long as 6 hours. It has been our practice to use only freshly autoclaved or boiled neopeptone water to eliminate any growth-lag.

(c) *Phagocytosis in Human Blood.*—

1. *Source of Blood.*—It has been shown that normal adult human blood contains specific opsonins for virulent pneumococci, but that these opsonins are absent in infant's blood (14). We have obtained similar results with virulent streptococci, so that, whenever possible, we have used defibrinated infant's blood to exclude the effect of such natural specific opsonins. Satisfactory results, however, may be obtained by using a mixture of equal parts of adult serum diluted eight times with saline, and washed human blood cells.

2. *Technique.*—1 drop from a capillary pipette (equivalent to about 0.03 cc.) of the secondary culture described above is added to 0.25 cc. of infant's blood in a pyrex glass tube; the tube is sealed and rotated for 30 minutes at 37°C. The tube is then flamed, broken open, and 1 drop of the contents smeared as a blood film on a glass slide. The dried smear is flooded with 2.0 cc. of Wright's stain,¹ and, after 6 minutes, 2.0 cc. of distilled water is added and left on for 4 minutes. The slide is then washed in running water and allowed to dry. Further details of the phagocytic technique are given in a paper by Ward and Enders (14). Counts are made of the number of organisms phagocytized by 25 or 50 polymorphonuclear leucocytes, and the percentage of these cells taking part in the phagocytosis is noted. The extracellular organisms are studied for the presence or absence of capsules.

(d) *Mouse Phagocytosis.*—Normal mice are prepared by injecting 0.5 cc. of saline into the peritoneum in order to induce a cellular exudate in advance of the injection of organisms. 4 hours later 0.25 cc. of the secondary culture of the variant to be investigated is injected into each mouse. One mouse is killed $\frac{1}{2}$ hour later, and a drop of the peritoneal exudate is withdrawn with a wide bore capillary pipette, smeared on a glass slide, and stained with Wright's stain as described above. A second mouse is killed after 1 hour, a third after 2 hours, a fourth after 4 hours, and the peritoneal exudate similarly smeared and studied for phagocytosis and encapsulation of free organisms.

(e) *Mouse Virulence.*—A 24 hour 5 per cent horse serum neopeptone water culture is serially diluted 10^{-1} to 10^{-6} in neopeptone water, and 0.5 cc. of each of these dilutions injected intraperitoneally into normal mice. 0.1 cc. of the 10^{-6} dilution is plated out on blood agar, and the number of colonies counted after incubation. The mice are observed for 4 days, and the cause of death verified by heart blood culture.

(f) *Spontaneous Agglutination.*—A culture in 5 per cent horse serum neopeptone water or in infusion broth without serum is incubated for 18 hours, well shaken up, and 0.5 cc. of this emulsion is mixed with an equal quantity of saline and placed in a water bath at 55°C. After 3 hours, the tubes are read as for agglutination.

¹ 0.3 gm. of powder, obtained from the Coleman and Bell Co., Norwood, Ohio, is added to 100 cc. of absolute methanol, and allowed to stand for 1 week, being shaken at intervals.

RESULTS

A description of the variants necessarily entails the use of a nomenclature. In so far as possible, we have tried to retain the terms of other writers, introducing new terms only when it seemed necessary. To avoid confusion, we have attempted to correlate our own with previous classifications.

In all, we have studied 75 strains, and have encountered four main variants. Two variants are virulent for man—the F and M variants. A third is an attenuated form of the M variant. The fourth, or C variant, is avirulent. The characteristics of each of these variants are given below. These have been summarized in Table I to facilitate comparison, and photographs (Figs. 1, 2, and 3) of the different colonies are included.

F Variant

We have isolated this variant from blood cultures in septicemias, from the throat in scarlet fever, from acute sore throats, from localized infections, and from normal throats. This variant is culturally stable, although occasionally the M variant develops spontaneously in meat tubes.

The F variant is not itself mouse-virulent, but if a large amount (1.0 cc.) of a 5 per cent horse serum neopeptone water culture is injected into the peritoneum of a normal mouse, the mouse may die. If death occurs within 2 days, the M or mouse-virulent variant is frequently recovered in pure culture from the heart's blood. With certain strains, however, the mouse may survive indefinitely, or die eventually with the F variant in the heart's blood.

This variant is resistant to phagocytosis in infant's blood, and small or fragmentary capsules are usually demonstrable in the blood films. If the organisms, in the phase of growth in which they resist phagocytosis in infant's blood, are introduced into the peritoneum of a prepared mouse, they are fairly resistant to phagocytosis during the first $\frac{1}{2}$ hour. At the end of an hour, however, practically all the cocci have been phagocyted. The few cocci that remain free show no capsular structure.

The colony on the special blood agar plates has an irregular contour, with a marked crater-like central depression (see Fig. 1). It grows

flocculently in 5 per cent horse serum neopeptone water. It agglutinates spontaneously in infusion broth without serum, but remains in even suspension in 5 per cent horse serum neopeptone water.

We have experienced difficulty in correlating the F with any previously described variant. It conforms most closely with the published description of Loewenthal's N variant (3), except that the N was found to be highly virulent for mice. Unfortunately, the single N culture which we were able to obtain from Germany did not conform to Loewenthal's description, and was apparently an M variant. It is likely that the F corresponds to Dawson and Olmstead's (4) smooth convex variant. Todd's classification does not include this form.

Atypical F Variant

This variant has been isolated from the blood stream of two cases of mastoiditis. It differs from the typical F only in having a conical colony without central depression, and forming heavier capsules. It may possibly be a transition form between the F and the M, since it has been isolated only in combination with the M.

M Variant

The sources of isolation include blood cultures from cases of septicemia, acute sore throats, localized infections, and normal throats. This variant is apt to change into the attenuated M variant when kept under laboratory conditions.

The M is more or less mouse-virulent, and the virulence, if not maximal, can be increased by mouse passage. This form is resistant to phagocytosis in infant's blood and in the mouse peritoneum. In each case, the cocci are surrounded by a well marked capsular structure. These capsules persist in the mouse, and the organisms increase rapidly in number as time goes on.

The appearance of the colony depends to some extent on the length of incubation time. The younger colonies—6 to 8 hours—are of the mucoid type, being smooth, watery, and of regular contour. The older colonies have a matt or wrinkled surface, but still retain the regular contour (see Fig. 2). The colonies are flatter and larger than those of the F or C variants. Growth in 5 per cent horse serum neo-

peptone water is diffuse, and there is no spontaneous agglutination in either plain broth or in the 5 per cent horse serum neopeptone water. This variant appears to be identical with the matt virulent variant of Todd (2), the M variant of Loewenthal (3), and the mucoid variant of Dawson and Olmstead (4).

Attenuated M Variant

This has been met with only in cultures which have been kept in the laboratory for some time. Some of the strains which are used for toxin production are of this type. Similarly, an old culture of *Streptococcus epidemicus* was an attenuated M. Unfortunately, we had no recent isolation from an outbreak of milk sore throat available for study, but the descriptions in the literature would lead us to believe that the *Streptococcus epidemicus*, when newly isolated, is probably an M variant.

It is not mouse-virulent, but according to Todd (2) the virulence can be restored by mouse passage. On the other hand, he states that, if cultivated for long periods on blood agar, it may give off a permanently avirulent variant. We believe that the latter is the C variant, described below.

The attenuated M does not resist phagocytosis in infant's blood or in the mouse peritoneum, although in both cases the capsular structure is morphologically indistinguishable from that of the M variant. One can often find capsulated forms within the cell.

The colony appearance is the same as that of the M. Its growth in 5 per cent horse serum neopeptone water may be either diffuse or flocculent. If grown in this medium it agglutinates spontaneously, but when grown in infusion broth without serum it remains in even suspension. It corresponds to the matt attenuated variant of Todd (2).

C Variant

We look upon this as the avirulent variant, although it was on one occasion isolated from a case of mastoiditis with sinus thrombosis, which ended in recovery. There were several negative blood cultures in this case, but one blood culture was positive, and from it the C variant was isolated in pure culture. The C was also found in normal

throats and in the glossy, avirulent cultures sent to us by Dr. Lancefield. It is not mouse-virulent, and according to Todd (2), this variant does not become virulent on mouse passage. It does not resist phagocytosis either in the infant's blood or in the mouse peritoneum, and has no capsular structure.

The colony that we have most frequently seen is conical in shape, without central depression (see Fig. 3); but occasionally we have noted a flat, rough colony form. It grows flocculently in 5 per cent horse serum neopeptone water, and agglutinates spontaneously in both infusion broth without serum and 5 per cent horse serum neopeptone water. It is not unlikely that it is this variant which causes so much trouble in agglutination tests.

The C appears to correspond to Todd's glossy avirulent variant (2), and perhaps, on the basis of avirulence, to Dawson and Olmstead's smooth flat (4).

Unclassified Variant

We have been unable to classify two strains which behaved in a similar manner. One of these was isolated from a normal throat, and the other from the blood stream in a fatal septicemia. There were two colony forms, one conical and the other mucoid. Single colony picks of either form constantly reproduced both types of colony. Growth in 5 per cent horse serum neopeptone water was flocculent.

There was abundant phagocytosis in both infant's blood and in the mouse peritoneum. In each case, some of the free cocci had no capsule, while others were heavily capsulated. The culture from the heart's blood, after mouse passage, again yielded the two colony types. This variant is apparently a very unstable M, but we prefer to leave it unclassified.

COMMENT

Although, as we have stated, our chief interest in this investigation centered around the question of virulence, it was first necessary to devise methods, which we have described, by which we could identify the different variants.

Recognition of Variants.—Our experience in studying numerous strains and their variants has taught us that no single method is abso-

lutely reliable. Dawson and Olmstead (4) have pointed out the value of using neopeptone in differential media, and we have used this as the basis of all our media. The colony form on neopeptone blood agar perhaps gives the most information of any one test, and with a little experience, the appearance of the colony is a fairly accurate guide in the majority of cases. The type of growth in serum neopeptone water is a useful confirmatory test, and in case of doubt we have found the phagocytic test to be reliable (with the possible exception of one strain) in distinguishing the virulent from the avirulent variants. The phagocytic test is particularly useful in distinguishing the M variant from the attenuated M variant.

Virulence.—Since the discovery that the hemolytic streptococcus formed an exotoxin, there has been a distinct tendency by certain writers to correlate virulence with the ability to secrete toxin (6). We do not agree with this view, but believe that virulence and toxigenicity are independent attributes of the hemolytic streptococcus. Eagles (7) has shown experimentally that animal virulence has no relation to toxin production, and our own observations bear this out, strains which are being used for toxin production having none of the criteria of virulence.

Menkin (8) has observed that there is delayed fixation of streptococci in the zone of inflammation in contrast to the prompt fixation of staphylococci. These experiments have been confirmed by Dennis and Berberian (9). This phenomenon is no doubt an important factor in the dissemination of this organism, but Menkin does not believe that it determines the virulence of the organism.

Tillett and Garner (10) have recently shown that hemolytic streptococci of human origin produce a soluble substance which lyses human clot, and this again may be a factor in virulence. We, however, have found that avirulent variants also produce this lytic principle, and as Tillett and Garner have pointed out, the filtrate of a mouse-virulent culture does not lyse mouse clot.

Although the foregoing properties of the hemolytic streptococcus are no doubt important supplementary factors, we do not regard any of them as the fundamental factor which determines the virulence of the organism. To us, this appears to depend upon the capacity of the organism to resist phagocytosis and consequent destruction by the

TABLE I

Variant	F	M	Attenuated M	C
Source	Blood stream, local lesions, inflamed and normal throats	Blood stream, local lesions, inflamed and normal throats	Laboratory cultures	Normal throats ? Blood stream (one case)
Virulence	Virulent for man, non-virulent for mice	Virulent for man, virulent for mice	Non-virulent for mice	Non-virulent for man, non-virulent for mice
Resistance to phagocytosis of young culture in infant's blood	Resistant	Resistant	Non-resistant	Non-resistant
Resistance to phagocytosis of young culture in mouse peritoneum	Somewhat resistant at first. Later, non-resistant	Resistant	Non-resistant	Non-resistant
Capsule formation in young culture	Small capsule	Large capsule	Large capsule	No capsule
Colony formation on special medium	Irregular, with central crater-like depression	Regular, flat, watery surface in young colony	Regular, flat, watery surface in young colony	1. Conical ? 2. Rough, flat
Growth in 5% horse serum neo-peptone water	Flocculent	Diffuse	Diffuse or flocculent	Flocculent

<p>Behavior of cultures after shaking up and incubating for 3 hrs. at 55°C.</p> <p>(a) Culture in 5% horse serum</p> <p>(b) Culture in broth</p>	<p>(a) Remains in suspension</p> <p>(b) Agglutinates spontaneously</p> <p>M variant or no change</p>	<p>(a) Remains in suspension</p> <p>(b) Remains in suspension</p> <p>M variant</p> <p>M of Loewenthal; matt virulent of Todd</p>	<p>(a) Agglutinates spontaneously</p> <p>(b) Remains in suspension</p> <p>M variant</p> <p>Matt attenuated of Todd</p>	<p>(a) Agglutinates spontaneously</p> <p>(b) Agglutinates spontaneously</p> <p>No change</p> <p>Glossy of Todd; ? smooth flat of Dawson and Olmstead</p>
<p>Variant produced by mouse passage</p>				
<p>Correlation with variants described in literature</p>				

cells of the host, as was demonstrated by Bordet (11) nearly 40 years ago. This author, in the year 1897, injected guinea pigs with virulent streptococci, and noted that the majority of the organisms were phagocytosed at once, but the few that remained free developed capsules, resisted phagocytosis, and increased in number until death of the animal ensued. This clue to the virulence mechanism of at least some strains of streptococci was largely forgotten until Hare (12) in 1929 showed that mouse-virulent streptococci in young culture resisted phagocytosis by human blood, whereas old cultures were readily taken up by the phagocytes. Seastone (13) has recently demonstrated that hemolytic streptococci which grow diffusely in serum broth resist phagocytosis in young culture, and he correlated this resistance with the development of a capsule. We have found that the M variant freshly isolated from human infections grows diffusely in serum broth, develops capsules in young cultures, and that these organisms resist phagocytosis. It is clear that both Hare and Seastone were working with the M variant.

On the other hand, the F variant, frequently isolated from human infections (some of them fatal septicemias), grows flocculently in serum broth, and when grown in young culture in the usual laboratory media fails to develop capsules and is readily phagocytosed. Both M and F variants, however, develop capsules and resist phagocytosis if they are grown for a short time in undiluted human serum. The same results may be obtained by substituting equal parts of horse serum and neopeptone water. This is an obvious convenience. The significance of the capsule in relation to virulence will be discussed later.

Although both the F and the M variants resist phagocytosis under these conditions, only the M variant has any virulence for mice. After much study, we are unable to explain the lack of virulence for mice in the case of the F variant. Its human virulence can hardly be questioned, since it has been repeatedly isolated from the blood stream in fatal septicemias.

On the basis of these facts, we have abandoned the mouse test in favor of resistance to phagocytosis as a criterion of human virulence. It might be argued that any streptococcus, virulent or avirulent, would resist phagocytosis under these experimental conditions, but the validity of the test is substantiated by: (1) the phagocytosis of the

attenuated M and of the C variants under the same conditions; and (2) the phagocytosis of the M and F variants in the presence of specific opsonin.

Dawson and Olmstead (4) have suggested that the mucoid (M) variant is responsible for the more fulminating streptococcal infections. Although we have isolated this variant from two such cases, an examination of all the cases we have studied leaves us in doubt as to whether the M variant is really more virulent than the F variant. For example, in one outbreak of puerperal sepsis, we isolated the F variant from the vagina and blood stream of one fatal case, the M variant from the vagina and blood stream of another fatal case, and also from the vaginae of two other cases which recovered without blood stream invasion.

We have made one observation which may possibly have some bearing upon the manner in which both the F and M variants maintain themselves in the tissues of the body. The organisms will grow out in undiluted human serum when the inoculum is taken from an ordinary serum broth culture, and as has been stated, the young culture in undiluted serum is resistant to phagocytosis. Subsequent cultivation, however, from serum to serum, is dependent upon the addition of cysteine to the serum. This suggests that a lowered oxygen tension in the tissues may be an important factor in the rate of streptococcal multiplication in the body.

Capsules.—The resistance to phagocytosis of the virulent variants appears to be associated with the presence of capsules on the organisms, better marked in the case of the M variant. No capsule can be demonstrated on the avirulent variant. However, this structural difference between the virulent and avirulent variants cannot be the sole factor in determining resistance to phagocytosis, since the attenuated M variant, which is readily phagocytized, has a capsule which is indistinguishable from that of the M variant.

Origin of the Different Variants.—At the present time, we are inclined to regard the F variant as the parent form of the *Streptococcus hemolyticus* of human origin, since we have only encountered it in primary isolation. On the other hand, all the other variants may be derived from the F. It may be of some significance that, while the F variant was isolated from the blood stream the whole year round, the

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EXPLANATION OF PLATE 23

- FIG. 1. Colony of F variant.
FIG. 2. Colony of M variant.
FIG. 3. Colonies of C variant.

M variant was only isolated in blood cultures during the winter months—the so called streptococcus season. Since it is known that the M variant may be derived from the F by mouse passage, it is conceivable that the winter prevalence of the M variant may be due to the high incidence of upper respiratory infections and consequent frequent passage of the hemolytic streptococcus from one case to another.

Other Variants.—In his account of the variants of the hemolytic streptococcus, Loewenthal (3) mentions two other variants, the O and the R. We have not encountered either of these two variants in our own work.

CONCLUSIONS

1. Four common variants of the hemolytic streptococcus of human origin have been described. These have been designated the F, M, attenuated M, and C variants.

2. The F and M variants only have been isolated from the blood stream in streptococcal infections. Only the M, however, has any primary virulence for the mouse.

3. Both these variants resist phagocytosis in human blood under suitable conditions, and this appears to be a reliable test for human virulence.

4. The attenuated M variant, found only in laboratory cultures, has a capsule as well developed as that of the virulent variants, and yet does not resist phagocytosis.

5. The C variant has no capsule and is readily phagocyted. It appears to correspond to the avirulent variant in other species.

6. An attempt has been made to correlate these four variants with those already described in the literature.

7. The application of these findings to the problem of virulence has been discussed.

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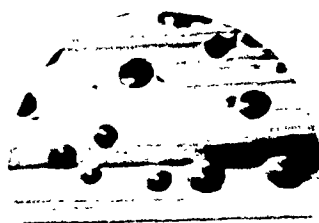
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EXPLANATION OF PLATE 23

FIG. 1. Colony of F variant.

FIG. 2. Colony of M variant.

FIG. 3. Colonies of C variant.



STUDIES ON THE HEMOLYTIC STREPTOCOCCUS OF HUMAN ORIGIN

II. OBSERVATIONS ON THE PROTECTIVE MECHANISM AGAINST THE VIRULENT VARIANTS

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In the preceding paper we have described the characteristics of two human-virulent variants (F and M) of the hemolytic streptococcus, and have presented the evidence which leads us to believe that the determining factor in the virulence of these two variants is their capacity to resist phagocytosis, although the exotoxin, the fibrinolysin of Tillett and Garner (1), and the delay in inflammatory fixation described by Menkin (2) may well be important accessory factors in human infections.

Were this conception of virulence correct, it should be possible to demonstrate opsonizing antibodies in the serum of the immune animal. Such opsonins were demonstrated by Denys and Leclef (3) in 1895, and by many other workers since that time. The difficulties of the phagocytic technique have discouraged extended studies of the opsonic antibody. In the accompanying paper we described a method of demonstrating the resistance to phagocytosis of streptococci in infant's blood; *i.e.*, in the absence of specific opsonin. In the present paper we are reporting the phagocytosis induced by adding opsonin to such a system, with studies upon the specificity and identity of the opsonin with other known antibodies to the streptococcus. The word "opsonin" is used throughout to connote that specific heat-stable antibody which combines with the organism to render it susceptible to phagocytosis; no distinction is drawn between the so called natural

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opsonins, immune opsonins, and bacteriotropins, inasmuch as we believe they are qualitatively identical (4).

It has long been recognized that the streptococci form an antigenically heterologous group, and many attempts have been made to type these organisms with antibacterial sera. The majority of workers have used agglutination, but, owing to the multiplicity of antigens involved, have met with many difficulties (5). Dochez, Avery, and Lancefield (6) in 1919 used agglutination and mouse protection in typing strains obtained from an epidemic of bronchopneumonia. On this basis, they were able to divide two-thirds of their strains into four groups, the remaining third being unclassified. More recently, Lancefield (7) has isolated the type-specific precipitinogen of the streptococci of human origin, designating it the M substance.

Since both the mouse protection and precipitin tests, although accurate, present considerable practical difficulties, as will be emphasized later, we have investigated the possibility of typing the streptococci by the opsonic technique. We have further attempted to identify the opsonin with the type-specific antibody which precipitates the M substance of Lancefield (7) and to differentiate the opsonin from the specific agglutinating antibody.

Experimental Procedures

(a) *Source of Material.*—The same as in Paper I.

(b) *Cultivation.*—The same as in Paper I.

(c) *Phagocytosis in Human Blood.*—The same technique was used as in Paper I, save that in experiments where the opsonins in immune sera were being studied 1 drop (0.03 cc.) of diluted serum was added to the system. We have found that foreign serum in strong concentration inhibits the phagocytosis of the streptococcus in human blood. Consequently, we have always diluted rabbit serum 1:10 and horse serum 1:5 before adding to the phagocytic system.

(d) *Preparation of Antisera.*—It has been the general experience of workers in this field that protective antisera are much more difficult to prepare than agglutinating antisera, and that living cultures must be used in the course of the immunization. This has also been the experience of Lancefield (7) in preparing sera which would precipitate the M substance. We have met with the same difficulty ourselves in preparing sera which contained the specific opsonins. This, indeed, has been our major handicap in attempting to type the virulent streptococci by the opsonic method. We have been unable to obtain opsonizing antisera by injecting cultures killed by heat, formalin, or alcohol. Even the injection of living cultures has been successful in only a few instances. Unfortunately, we realized too late the difficulties inherent in the production of these antisera, and there was then no time to

subject animals to the prolonged course of immunization which is apparently necessary. The method employed to produce active antiserum was as follows: 0.1 cc. of a 3 hour culture of the M variant in 20 per cent rabbit serum neopeptone water was injected intravenously into rabbits at weekly intervals. It was observed that the rabbits, in almost all cases, had a positive blood culture for from 1 to 3 days after this injection. In many rabbits we were unable to demonstrate the development of opsonins even after many weeks, during which this phenomenon had been repeatedly observed. We have not used the living F variant as an antigen.

(e) *Preparation of Bacterial Extracts.*—Extracts were prepared from 16 hour broth cultures of the F and M variants exactly as described by Lancefield (7).

(f) *Absorption of Antisera.*—All absorptions were carried out according to the technique of Lancefield (7), 16 hour broth cultures of the F and M variants being used.

(g) *Precipitation Tests.*—The bacterial extracts were layered on the unabsorbed and absorbed antisera and read after 2 hours at room temperature. Some of our antisera gave a positive ring test with broth or peptone alone. To avoid error from this source, it is necessary to wash thoroughly cultures which are to be used for the preparation of bacterial extracts. This phenomenon made it impossible in most cases to investigate any bacterial precipitinogens in the supernatant fluid of broth cultures.

(h) *Agglutination Tests.*—We have used two techniques in preparing cultures for agglutination tests: (1) Young cultures of the M and F variants in 50 per cent horse serum neopeptone water were prepared as for the phagocytic test. (2) Cultures of the M and F variants were grown in 5 per cent horse serum neopeptone water for 15 hours after the first visible sign of growth.

In both cases, the cultures were well shaken up and 0.5 cc. added to an equal quantity of saline dilutions of the antiserum in agglutination tubes, the control tube for spontaneous agglutination containing culture and saline alone. These tubes were placed in the water bath at 55°C., and read at the end of 3 hours.

(i) *Bactericidal Technique.*—These tests were carried out by the modified Todd technique described by one of us (8). 16 hour cultures of the F and M variant in 5 per cent horse serum neopeptone water were serially diluted in neopeptone water, and added to the mixture of blood and diluted antiserum. To a control series, the same dilution of normal serum was added. The tubes were sealed, rotated in a 37°C. incubator for 48 hours, and then 1 drop of the contents of each tube plated out.

(j) *Passive Mouse Protection.*—A series of six mice were injected intraperitoneally with 0.2 cc. of a homologous antiserum, another series with 0.2 cc. of a heterologous antiserum, and a third series with 0.2 cc. of normal rabbit serum. The next day, each series was injected intraperitoneally with the living, virulent culture, one mouse receiving 0.5 cc. of the culture diluted 1:10, the next mouse receiving 0.5 cc. of a 1:100 dilution and so on, the sixth mouse in the series receiv-

ing 0.5 cc. of a 10^{-6} dilution of the culture. Mice which died before the end of the 4th day were autopsied and cultures made of the heart's blood. Mice which survived 4 days were assumed to be protected.

(k) *Phagocytosis in Mouse Protection Test.*—Phagocytosis in infant's blood of a young culture of the organism in the presence of the homologous antiserum, of the heterologous antiserum, and of the normal serum were carried out in the usual way. In addition, one mouse was injected intraperitoneally with 0.2 cc. of a homologous antiserum, and one mouse with 0.2 cc. of a heterologous antiserum; the next morning, each mouse received an intraperitoneal injection of 0.5 cc. saline, and 4 hours later an intraperitoneal injection of 0.01 cc. of a young culture of the organism grown in 50 per cent horse serum neopeptone water, centrifuged and resuspended in neopeptone water. The mice were killed 4 hours later; the peritoneal contents smeared on a glass slide, and stained with Wright's stain.

RESULTS

(a) *The Correlation between Mouse Protection and Phagocytosis.*—It is well known that mice can be protected against virulent hemolytic streptococci (M variant) by a previous injection of a specific antiserum. If this protection is due, or mainly due to the phagocytosis of the injected organisms, it is necessary to show that the protecting antiserum contains specific opsonin, and that phagocytosis actually occurs in the mouse in the presence of this serum.

Tables I and II show clearly that Antiserum 301 contains opsonin for the Strain S₂₃ and protects mice against this strain, whereas Antiserum 190 and normal rabbit serum neither protect the mice nor opsonize the organisms.

If the peritoneal fluid of the mouse is studied 4 hours after 0.01 cc. of a young culture is injected, we have observed that in the case of the mouse receiving the specific antiserum, free cocci either cannot be found or are few in number, whereas many of the cells contain cocci, some of which stain faintly, suggesting that digestion has already commenced. On the other hand, in the fluid of the mouse receiving the heterologous antiserum, there are an enormous number of free, encapsulated streptococci, with no evidence of phagocytosis.

(b) *The Opsonic Action of Anti-M Sera on F and M Variants.*—We had only two opsonizing antisera for strains of which we had both the F and M variants. The opsonic experiments performed with these two antisera on the F and M variants of homologous and heterologous strains are recorded in Table III. In this experiment, Anti-

serum 189 was prepared by injecting a rabbit with living Phil. (M) culture and Antiserum 190 was prepared by injecting a rabbit with living Men. (M) culture. The strain C. H. was found to be opsonically homologous with the strain Phil. and is included in the experiment to demonstrate this method of typing. The strain Col. was not opsonized by either antiserum.

TABLE I

Dose of streptococci Strain Sz-M variant	Fate of mice injected with		
	Antiserum 301	Antiserum 190	Normal serum
10 ⁻¹	D*	D	D
10 ⁻²	S	D	D
10 ⁻³	S	D	D
10 ⁻⁴	S	D	D
10 ⁻⁵	S	D	D
10 ⁻⁶	S	D	S

* In this table, D = death of mouse, S = survival of mouse.

TABLE II

No. of streptococci (Strain Sz-M variant) phagocyted by infant's blood plus		
Antiserum 301	Antiserum 190	Normal serum
291-100*	0-0	0-0

* In this and subsequent tables, the first figure represents the number of organisms phagocyted by 25 polymorphs, the second figure the percentage of polymorphs which have phagocyted one or more organisms. Thus, 291-100 indicates that 291 streptococci were counted in 25 polymorphs, and that no empty cells were met with.

(c) *The Relationship of the Specific Opsonin, the Specific Precipitin, and the Specific Agglutinin in Anti-M Variant Sera.*—In the experiment reported in Tables IV and V, we have tested for the specific opsonin, the specific precipitin, and the specific agglutinin in an antiserum unabsorbed, absorbed with the homologous atypical F variant, and absorbed with the homologous M variant. The experiment is controlled by absorbing the same antiserum with heterologous F and M variants. The whole experiment, including the absorptions, was

ing 0.5 cc. of a 10^{-6} dilution of the culture. Mice which died before the end of the 4th day were autopsied and cultures made of the heart's blood. Mice which survived 4 days were assumed to be protected.

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variant has retained this antigen or at least has retained it in part. Owing to the difficulty of centrifuging young cultures of the F variant, it has proved technically impossible to absorb serum with young F organisms.

TABLE IV

Serum	Agglutination							Precipitation
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	
Unabsorbed ss. M	++++	++++	++++	++++	+++			
" " F	++++	++++	++++	++++	+++	0	0	++++
Homologous F-absorbed ss. M	0	0	0	0	0	++	0	++
" " F	+	0	0	0	0	0	0	+++
Homologous M-absorbed " M	0	0	0	0	0	0	0	+
" " F	0	0	0	0	0	0	0	0
Heterologous F-absorbed " M	++++	++++	++++	++++	0	0	0	0
" " F	++++	++++	++++	++++	+++	+	0	++++
Heterologous M-absorbed " M	++++	++++	++++	++++	++++	++	+	+
" " F	++++	++++	++++	++++	0	0	0	++++
						+	0	+

TABLE V

Serum	Phagocytosis of M variant*						
	Concentration of serum						
	1:80	1:160	1:320	1:640	1:1280	1:2560	0
Unabsorbed	1063-86†	636-64	270-30	177-18	38-8	20-4	10-2
Homologous F-absorbed	920-80	425-48	155-16	47-6	38-6	16-6	10-2
" M-absorbed	21-2	7-4	45-6	10-4	22-4	38-10	10-2
Heterologous F-absorbed	617-66	592-48	286-34	94-14	34-4	20-4	10-2
" M-absorbed	877-80	429-44	237-26	68-12	20-6	41-6	10-2

* In another experiment the F variant was used as well as the M variant, with essentially the same result.

† In this experiment 50 cells were counted instead of 25 cells.

The conception that the specific agglutinin is antigenically different from the specific opsonin, was further strengthened by the isolation of a strain whose mucous (M) variant was agglutinated to titer but not opsonized by an antiserum known to possess both specific agglutinin and specific opsonin for its homologous strain. This

repeated with essentially the same findings. Attention should be drawn to the use of an atypical F variant (see Paper I) in the homologous absorption. This was unavoidable, since we had no antiserum strong enough to use with a homologous typical F variant.

Assuming that the results would be the same had a homologous typical F variant been used, the experiment recorded in Tables IV and V shows that: (1) The homologous M variant absorbs completely the specific opsonin, specific precipitin, and the specific agglutinin from the antiserum for both itself and the F variant. (2) The homologous F variant absorbs the specific agglutinin, but leaves unabsorbed the major part of the specific precipitin and the specific opsonin. (3) Neither the heterologous F variant nor the heterologous M variant

TABLE III

Strain	Variant	No. of streptococci phagocyted by infant's blood plus		
		Normal serum	Antiserum 189	Antiserum 190
Phil.	F (atypical)	6-8	217-88	4-4
Phil.	M	2-4	355-100	2-4
CH.	F (atypical)	18-20	238-80	21-24
CH.	M	0-0	92-40	0-0
Men.	F	0-0	4-4	96-52
Men.	M	0-0	0-0	51-28
Col.	F	0-0	6-4	0-0
Col.	M	0-0	0-0	0-0

appreciably absorb the specific agglutinin, the specific precipitin, or the specific opsonin.

These findings suggest that: (1) The specific opsinogen is identical with all or part of the specific precipitinogen (the M substance of Lancefield (7)). (2) The specific agglutinin is not antigenically identical with the specific precipitinogen or the specific opsinogen.

The previous experiment reported in Table III indicates that the F and M variants in young culture have the same opsinogen, and the only explanation we can offer for the failure of the F variant in an old culture to absorb the specific precipitin and the specific opsonin is that, in the old cultures used for absorption, the F variant has lost its specific opsinogen and specific precipitinogen, whereas the M

(d) *Opsonic Typing of the Hemolytic Streptococci*.—This phase of the work was severely handicapped by our difficulties in producing opsonizing antisera, but the five available sera indicated the immensity of the problem. One antiserum identified two homologous strains out of twenty-two examined; two antisera did not identify any strains out of twenty-four examined. Investigation of two commercial polyvalent antibacterial sera revealed no effective concen-

TABLE VII

Strain	Variant	Phagocytosis in the blood of					
		Infant	H.K.W.	C.L.	J.H.M.	F.B.G.	J.F.E.
Men.	F	0-0	2-4	69-28	0-0	122-72	14-16
"	M	0-0	0-0	0-0	0-0	16-16	0-0
W	"	0-0	104-72	0-0	0-0	0-0	0-0
S ₂₁	"	0-0	34-32	2-4	12-8	126-76	—
SR	F	0-0	0-0	14-6	38-24	14-20	54-40
Cal.	"	0-0	0-0	41-24	4-8	2-4	155-60
FL	M	0-0	2-4	2-4	0-0	4-4	0-0

TABLE VIII

Strain	Variant	Infant's blood	Concentration of adult's serum	Phagocytosis
		α.		
Phil.	M	0.25	1:8	147-84
"	"	0.25	1:16	52-72
"	"	0.25	1:32	40-32
"	"	0.25	1:64	21-20
"	"	0.25	1:128	5-4
"	"	0.25	—	0-0

tration of opsonins for any of forty-seven strains examined, thus confirming similar work by Hare (11) and by Fothergill and Lium (16).

(e) *Natural Opsonins*.—During the course of these experiments, an M variant was isolated from the throat of one of us during an attack of acute pharyngitis. Some months after convalescence, and long after the organism had disappeared from the nasopharynx, this individual's blood phagocyted this organism readily, whereas we were unable to demonstrate any phagocytosis of this organism by the blood

of any other adult in the laboratory. Seastone (9) has reported a similar experience, using essentially the same phagocytic technique. These observations would appear to explain the absence of opsonins to virulent streptococci in infant's serum, and the presence of such opsonins for certain strains in the serum of adults. On this account, we would prefer the term "acquired opsonins," although to avoid confusion we have retained the more commonly used term "natural opsonins" in this section.

In Table VII, we have recorded the phagocytic effect of natural opsonins on certain F and M variants. This experiment shows that opsonins are absent in infant's serum and present in varying amount for certain strains in the serum of adults.

Inasmuch as polyvalent antisera do not contain opsonins for virulent streptococci, we have titrated the natural opsonin in the serum of one adult in order to determine whether it was sufficiently concentrated to use in immunotransfusion. This titration is recorded in Table VIII. In theory, one might expect to benefit a child by transfusing him with 500 cc. of whole blood, containing natural specific opsonin.

DISCUSSION

As far as is definitely known at the present time, the body can destroy Gram-positive organisms in only one way—by their phagocytosis and intracellular digestion. In the first paper, we presented evidence showing that the virulence of the hemolytic streptococcus was correlated with the resistance of the organism to phagocytosis. In this paper, we have presented evidence for the truth of the corollary; *i.e.*, that immunity is correlated with the presence in the animal of specific opsonizing antibody against the virulent variants.

Although Day (12) has recently questioned the type specificity of streptococcus immunity, our own experiments and those of other workers in this field indicate that immunity is strictly type-specific. In view of the accumulating evidence that it is possible to achieve a low level of non-specific immunity to infection with the pneumococcus, we regret that we did not have the opportunity of repeating Dr. Day's experiments with the cultures which he so kindly sent us. In the present state of our knowledge, however, we feel that it is wiser to emphasize the importance of type specificity in streptococcal immunity.

The determination of the types of streptococci has been attempted by three methods: agglutination, precipitation, and mouse protection tests. We do not believe that any of these methods is suitable for the rapid typing of freshly isolated strains. The agglutinative reaction has been shown by Andrewes (5) and Williams (13) to depend upon a multiplicity of antigens and antibodies, necessitating the absorption of antisera. We have presented evidence, moreover, that the agglutination of old cultures of virulent streptococci does not necessarily identify the all-important opsinogen of the young, capsulated organism. The capsulated streptococci in young cultures apparently resist agglutination. It appears to us that the protective mechanism is dependent upon the presence of a specific opsonin capable of combining with the capsular material surrounding the young, virulent streptococcus, and is independent of those antibodies which combine with the somatic antigens and which may be demonstrated by agglutination of older cultures.

The precipitation technique of Lancefield (7) is accurate, but, again, requires the preparation of bacterial extracts and the absorption of antisera before results can be obtained.

The mouse protection test has the disadvantage of requiring strains of high virulence for mice, and many freshly isolated human-virulent strains may require many passages through mice before they attain this degree of virulence.

In proposing the opsonic method for typing the human-virulent variants of the hemolytic streptococcus, we believe that it is more pertinent than the agglutination test and is simpler and more rapid than the precipitation and mouse protection tests. Perhaps the main reason for the desuetude into which the opsonic method has fallen in recent years is the difficulty of interpreting the results. When we carried out the test in the ordinary way with 18 hour broth cultures, we too were often unable to detect the action of a specific opsonin, since even infant's blood can phagocyte many cocci in a culture of this age and the addition of a specific opsonin does not make a really decisive difference in the amount of phagocytosis. When, however, the virulent streptococcus is grown for a short time only in 50 per cent horse serum neopeptone water, practically no phagocytosis takes place in infant's blood, and the addition of specific opsonin has now a decisive

effect, giving results easy to interpret, as will be seen by referring to Table III.

Our difficulties have not been with the opsonic method, but with the preparation of opsonizing antisera. Lancefield (7) has reported similar difficulties in preparing the type-specific precipitin, and Williams (13) states that the "proportion of rabbits yielding good protective antiserum is not great." All are agreed that living cultures must be used in the course of immunization. One might suppose that living cultures have to be used in preparing antisera, because any agent that kills the coccus alters the protein capsular substance (probably identical with Lancefield's M substance), so that it is no longer antigenic. And one could draw an analogy with the anthrax bacillus, for here again living cultures have to be used in immunizing animals, and Tomcsik and Szongott (14) state that the capsular material of the anthrax bacillus is protein in nature. But against such a theory must be set the fact that mice can be actively immunized against the M variant of the hemolytic streptococcus by injecting the heat-killed organisms (15).

In view of the difficulty experienced in preparing effective antisera against the hemolytic streptococcus in laboratory animals, it is not surprising that the polyvalent antibacterial sera available for the treatment of streptococcal infections have proved valueless. Both Hare (11) and ourselves have been unable to demonstrate opsonic antibodies in such sera. Fothergill and Lium (16) report the absence of bactericidal antibodies in these sera. It would seem logical to attempt to produce effective opsonizing antisera against the commoner types of hemolytic streptococci, although it is true we have no evidence that they would be therapeutically successful. Such evidence might be obtained in another way. We have shown that adults have specific opsonins against certain strains of streptococci, and it would be practical to select a donor having the specific opsonin for transfusion of patients with streptococcus septicemia.

These studies are neither as thorough nor as complete as we would wish, but the work had to be discontinued at this point and the papers are published in the hope that the data may be of some assistance to other workers in this field.

CONCLUSIONS

1. An antiserum which specifically protects mice against a virulent culture (M variant) of the hemolytic streptococcus contains specific opsonin. Phagocytosis of the organisms can be observed in the peritoneum of the protected mouse.

2. An antiserum prepared by injecting an animal with the living M variant specifically opsonizes both the F and the M variant of the strain.

3. Evidence is presented which indicates the probable identity of the specific opsonin and the anti-M precipitin of Lancefield (7). Agglutination appears to be dependent upon a different antibody.

4. It is possible to type the hemolytic streptococci by means of specific opsonins, and the opsonic method has certain advantages over agglutination, precipitation, and mouse protection tests. It is evident from what little has been done that there are many types.

5. The serum of infants contains no opsonin for the virulent hemolytic streptococci, but the serum of adults may contain specific opsonins for certain strains. Inasmuch as no opsonins were demonstrable in two polyvalent antibacterial sera examined, the possibilities of therapeutic transfusion are discussed.

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STUDIES ON MENINGOCOCCUS INFECTION

VII. THE STUDY OF AN ISOLATED EPIDEMIC

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The previous studies from this laboratory of meningococcus infection, particularly those on the carrier problem (1), had left certain important questions unanswered which from their nature could best be investigated during a localized epidemic. Among such questions was the duration of the carrier state when this was due to known "epidemic" or disease-producing strains as contrasted with the presumably saprophytic or avirulent strains met with in the case of groups of healthy persons studied and reported in the earlier paper (1). Further, the whole question of the exact nature of the carrier state in the case of the meningococcus was doubtful. Was it to be looked upon as a true infection of the body by the organisms, albeit more or less localized to the nasopharyngeal mucous membrane, or was the relationship between meningococcus and host of much less intimate nature involving no reaction of the host to the organism? It was certainly desirable to obtain closer information as to the virulence of strains, epidemic or sporadic, from the spinal fluid or the nasopharynx, than had hitherto been available. The latter investigation depended, of course, on the introduction and perfection of a suitable test for meningococcus virulence in experimental animals.

With the unanswered questions in mind an invitation from Colonel Addison Davis of the 6th Corps Area Headquarters, Chicago, to participate in the investigation of an outbreak of cerebrospinal meningitis in a Civilian Conservation Corps lumber camp in Wisconsin was gratefully accepted.¹

¹ My thanks are due to all those with whom I worked whose efforts made such detailed work possible, especially to Colonel Addison Davis, to Major A. P. Hitchens, to Lieutenant B. L. Camp, to Lieutenant T. Ennis, to Dr. W. D. Stevall,

Investigation commenced on Feb. 22, 1934, at C.C.C. Camp Rusk, Wisconsin, a camp with a personnel of 215, situated 25 miles from the nearest town. The population of the latter was approximately 3,500. The history of the epidemic up to that time was as follows: On Nov. 25, 1933, a case of cerebrospinal meningitis occurred in a C.C.C. worker. Since that time 5 other cases had occurred in the camp and 8 in the civilian population of about 6,000 persons. In point of time the cases had been well scattered, occurring every 1 or 2 weeks between Nov. 25 and the date that the investigation was commenced. The camp had been intermittently in quarantine, but before this was first applied and at intervals thereafter contact between the camp and the civilian population had been very close. No strains of meningococci had been recovered by the military authorities working with a mobile laboratory, but Dr. Stovall of the Wisconsin State Board of Health had isolated 2 strains and identified them as "Type III" meningococci. During the 32 days spent at the camp by the present author only 2 fresh cases occurred. Any problems, therefore, which were connected with cases of the frank disease itself could not be investigated and attention was paid to the carrier situation.

In all, 72 Type I-III and 23 Type II and 2 Type VII carriers were found in the camp. A few were found among the civilian population but, unfortunately, circumstances did not allow a detailed examination for carriers outside the camp. The work in the camp was necessarily confined, for the most part, to the study of the carriers, the approximate degree of their infection and the length of duration of the carrier state. This was supervised personally for a little over 4 weeks and was then carried on for another 6 weeks first by Major A. P. Hitchens and later by Dr. Ernest Q. King, both of whom have kindly given permission for the inclusion of their records in this paper.

In addition to the work carried on at the camp itself, cultures of carrier strains and samples of blood from the carriers were obtained and were brought back to the laboratories at The Rockefeller Institute where further investigations were made. It is realized that the results with the carrier and case strains, especially with regard to the virulence tests, are somewhat unsatisfactory on account of the interval elapsing between the isolation of the strain and the carrying out of the virulence test. The arrangements made, however, were the best possible under the circumstances.

RESULTS

Of the 94 carriers 71 carried Type I, 22 carried Type II and 1 carried Type VII; one was a Type I carrier that once showed a Type II, and another, that once showed a Type VII; one, a Type II carrier once showing Type I; and one carrier showed Type II and Type VII

to Dr. W. Smith, to Dr. L. M. Lundmark and to C.C.C. enrollees Edmund Malecki and Sigfred Moinichen.

each once. This gives the high rate of 33.5 per cent Type I carriers and 10.7 per cent Type II. As far as throwing light on the duration of the carrier state is concerned, the results are unsatisfactory. Owing to the need for closing the camp as soon as possible, the authorities transferred the men or sent them home very early—in some cases even when the last swab had been positive for meningococci. Many other carriers were still positive, or had been so recently, when the investigation closed. As has been shown elsewhere (1), carriers may still be found positive even after $4\frac{1}{2}$ months of negative swabbings. This being so, it could not, of course, be said that any of these carriers cleared up, the total duration of the investigation being only $2\frac{1}{2}$ months. It seems probable that the clearing up of the carrier state should be based on negative weekly swabs lasting over a period of at least 2 or 3 months, since 3 consecutive negative weekly swabbings are of little value when assessing the normal duration of the carrier condition (1). Even if, however, the basis of 3 consecutive weekly swabbings should be adopted as indicating a cure, the results obtained in the present investigation were unsatisfactory. Thus:—

11 Type I } carriers were still positive when swabbing ceased.
4 Type II }

6 Type I } carriers showed only 1 negative culture when swabbing ceased.
8 Type II }

20 Type I } carriers showed only 2 negative cultures when swabbing ceased.
4 Type II }

5 Type I } carriers showed only 3 negative cultures when swabbing ceased and
4 Type II } the period from the last positive to the last negative was less than
3 weeks.

It is clear, therefore, that 42 out of 72 Type I carriers and 20 out of 23 Type II carriers, on any of the criteria accepted for the "cure" of meningococcus carriers, were still potentially affected when the investigation ended.

It is with these considerations in mind that the table showing the duration of the carrier state during epidemic conditions at Camp Rusk should be examined (Table I). It will then be seen that, on

the basis of "cure" constituted by 3 consecutive weekly negative swabs, 37.5 per cent of Type I carriers lasted 4 weeks or less, 33.5 per cent lasted 5 weeks or over and 29.3 per cent were still potentially affected when last seen, having lasted 4 weeks or less up to that time. With the Type II carriers 13 per cent lasted 4 weeks or less, 4.3 per cent (this represents only 1 case) 5 weeks or more and 82.7 per cent were still potentially affected when last seen.

TABLE I

Duration of carrier state	Type I carriers		Type II carriers	
<i>days</i>		<i>per cent</i>		<i>per cent</i>
1	16	22.2	2	8.7
1++	7	9.7	14	60.9
<i>wks.</i>				
1++			4	17.4
2	6	8.3		
2++	4	5.6	1	4.3
2½	1	1.4	1	4.3
3	3	4.2		
3++	10	14.0		
4	1	1.4		
5	2	2.8		
5++	5	7.0		
6++	7	9.7		
7++	2	2.8		
7½++	2	2.8		
8	1	1.4		
8½++	3	4.2		
9++	1	1.4		
11++	1	1.4	1	4.3

++ = negative swabbings not obtained 3 times at weekly intervals in these carriers.

Even on this basis, then, the number of Type I carriers remaining positive for over 5 weeks was high. The figure was comparable to that recorded by Flack (2). He showed that 47.59 per cent of contact carriers lasted over 4 weeks and 52.41 per cent under 4 weeks. In non-contacts the average duration was shorter and the figures were 27.77 per cent and 72.23 per cent respectively. The experience in this laboratory has been contrary to that of Flack in that non-contacts, carrying atypical strains, tend to be chronic carriers (1).

It is believed that the average duration of the carrier state as demonstrated at Camp Rusk or as recorded anywhere in the literature is put somewhat, and probably much, too low. In the case of the Camp Rusk epidemic another variable factor occurred in the change of personnel which took place twice during the investigation. Although exactly the same equipment be available and the same technique be adopted in each case, there is no doubt that the personal element is very great in any method used for the detection of meningococcus carriers. The individual who has had several years of close experience with this work will inevitably find a higher percentage of positive throat plates than anyone coming to the task relatively inexperienced.

It is undoubtedly true that the greater number of these carriers at Camp Rusk, carrying typical Type I or Type II (epidemic) strains, did not give such constantly positive plates as had been usual amongst the non-contact group carrying, for the most part, strains not identical with Gordon's 4 types; that is, what might be called non-epidemic strains (1). This fact, added to the fact that the demonstrable carrier duration with epidemic strains was relatively short, might be taken in support of the theory that virulent strains of meningococci are carried for only short periods as compared with the comparatively non-virulent or even saprophytic strains. This would be in accord with the known facts with regard to pneumococcus carriers (3-5). On the other hand, it must be borne in mind that laboratory conditions at the C.C.C. camp were by no means ideal and that this fact together with the changing personnel would undoubtedly affect the work adversely and produce a higher percentage of apparently negative throat cultures than was actually the case. The question of duration of the carrier state when virulent (epidemic) typical meningococcus strains are concerned, which is an important unsolved problem, must still be regarded as *sub judice*.

Virulence of Strains for Mice

A study of the virulence of meningococcus strains gathered during an epidemic depended, of course, entirely on the existence of a suitable animal test. Such a test had not been available, at least in small laboratory animals, until Miller (6) described his method of producing experimental meningococcal infections in mice by the use of mucin

suspensions. A study of this method confirmed all that Miller had claimed for it and showed that, at least in certain susceptible breeds of mice, it gave consistent results which could be used as a basis for the titration of meningococcal virulence.²

The following has been adopted as the standard method for the titration of the virulence of meningococcus strains. A 6 per cent solution of hog's gastric mucin at pH 7.4 is prepared according to the method described by Miller. The strain to be investigated is grown for 16 to 18 hours on a 10 per cent rabbit's blood pneumococcus agar. It is then washed off in normal saline and the saline suspension diluted until a standard suspension, containing 2,000,000,000 organisms to the cubic centimeter, is obtained. Dilutions of this suspension, each 1 in 10, are made in the 6 per cent mucin. For most strains it has been found that dilutions from 10^{-2} to 10^{-6} are sufficient for the titration of virulence. A cubic centimeter of 10^{-2} dilution will contain 20,000,000 organisms and a cubic centimeter of 10^{-6} , 2,000 organisms. 1 cc. of the 6 per cent mucin suspension of each dilution is injected intra-abdominally into at least 2 mice. It is important that all steps involving the meningococci, from the time they are washed from the plate until the inoculation is made, be carried out as rapidly as possible. The lowest dilution at which all mice die is taken as the end-point, but any deaths beyond this point are indicated by a + sign. Thus, if all mice die at 10^{-5} and only 1 out of 2 or 3 at 10^{-6} , the virulence is given as $10^{-5}+$. It has been found that in order to obtain consistent results the susceptible breed of white-face mice is the most satisfactory of those yet tested.

Using this method of virulence titration, 34 strains isolated from the carriers at Camp Rusk were tested after they were brought back to the laboratory at the Institute. Of these strains, 14 were Type I, 12 Type II, 3 Type VII and 5 were *Neisseria catarrhalis* strains isolated from carriers. Table II shows the mouse virulence of these strains.

It is clear from Table II that the majority of Type I strains had a high virulence, 78.6 per cent showing a virulence of 10^{-5} or greater. There is no explanation why one Type I strain should have failed to kill at all. At the time it was titrated it had been isolated from the nasopharynx 24 days, and the average elapsed time for Type I strains was 24.7 days with a spread of from 15 days (strain with virulence $10^{-5}+$) to 36 days (strain with virulence $10^{-5}+$). The virulence of

² Not all batches of mucin obtained from commercial sources are alike in their power of enhancing the pathogenic activity of meningococcus strains. Some batches of Wilson's gastric mucin, for example, fail to favor the organism at all.

the Type II strains is significantly less than that of the Type I. 66.7 per cent of Type II strains have a virulence of $10^{-4}+$ and only 1 strain (out of 12) has a virulence greater than this. The virulence of the atypical Type VII strains was much less, the most virulent strain being only $10^{-2}+$, while the strains of *N. catarrhalis* were completely avirulent with the exception of one that killed at 10^{-3} .

The high virulence of Type I strains and the moderately high but lesser virulence of Type II strains are interesting. It seems probable that the epidemic was produced by Type I or Group I-III strains.

TABLE II

Virulence	Type I		Type II		Type VII		<i>N. catarrhalis</i>	
	Strains	Per cent	Strains	Per cent	Strains	Per cent	Strains	Per cent
10^{-6}	4	28.6						
$10^{-5}+$	4	28.6	1	8.3				
10^{-5}	3	21.4						
10^{-4}			8	66.7				
$10^{-3}+$	2	14.3	1	8.3				
10^{-3}			1	8.3			1	20.0
$10^{-2}+$			1	8.3	1	33.3		
10^{-2}					1	33.3		
None	1	7.1			1	33.3	4	80.0
Average time between isolation and testing of strains, days.....	24.7		21.0		29.7		27.8	

In favor of this are the following facts. Both cases occurring during the personal investigation of the epidemic were Type I cases and one of them occurred in a known Type I carrier; Dr. Stovall had isolated 2 Type III strains at an earlier date; one of the cases proved later to be a Type I carrier, being found positive once 5 months after the onset of the meningitis (this is of doubtful significance; it may have been only a new infection from contact with other carriers); the Type I carrier rate was 33.5 per cent which is well over the "explosion point" of 20 per cent. On the other hand, it is possible that some cases may have been due to Type II organisms. One case, that was seen very shortly after the meningitis had disappeared, never showed any Type

I organisms in the nasopharynx but showed a Type II strain once 40 days after the onset of the disease; a specimen of spinal fluid taken from a civilian case at the height of the illness proved later to have precipitinogens which reacted moderately with Type II antimeningococcus serum (unfortunately no strain had been isolated from this patient at the time she was sick but her blood, taken 3 weeks after the onset of the disease, showed Type II agglutinins and protective antibodies as described below); finally, the Type II carrier rate of 10.7 per cent, while not very high, is a high normal figure and might be compatible with the appearance of a few Type II cases. The relative virulence of the 2 types as isolated from carriers might also be taken as suggestive of a main Type I epidemic with some Type II cases.

The Nature of the Carrier State

The nature of the carrier state in meningococcus infections has stirred up much controversy. The fundamental question at issue is whether this can be looked upon as a true infection or not. Are we to believe that the meningococcus on being implanted on the mucous surface of the nasopharynx causes any reaction within the host either local in the way of inflammation or general in the way of serological and other changes, or are we to look on the meningococcus carrier state as a more or less passive symbiotic relationship much like that of the *Staphylococcus albus* on the skin or Döderlein's bacillus in the vagina? Although some investigators (7) have claimed that meningococci usually, if not invariably, cause an inflammation of the mucosa of the nasopharynx, and although reports of epidemics of meningococcal nasopharyngitis preceding or running concurrently with epidemics of meningitis exist, nevertheless these are usually discounted and apparently the feeling of the majority of workers, whether published or not, is that the meningococcus produces no reaction in the mucosa and that the carrier state in this disease is not to be looked upon as an infection.

In the case of other organisms carried, as is the meningococcus, in the nasopharynx, there is the same lack of unanimity of opinion as to the precise status of the carrier condition. With the pneumococcus, however, there is some evidence from two different angles that the

carrier state is in the nature of an infection. Thus, Webster and Hughes (5) concluded that the numbers and degree of infection of pneumococcus carriers varied directly with the incidence of coryza, sore throat and other respiratory tract diseases. They thought that pneumococcus carriers are infected individuals with organisms growing in their nasopharyngeal tissues, and further that the presence or absence of the carrier state depended on the resistance of the host, a factor which varied with the season of year. Bull and McKee (8) had shown that a certain number of rabbits which gave no sign of infection following intranasal instillation of pneumococci developed protective antibodies and that their serum contained agglutinins and fixed complement. Neufeld and Tulczynska (9) also were able to show that mice inoculated intranasally with pneumococci and becoming carriers acquired an immunity which was higher the longer the carrier state persisted. They thought that the immunity might be due to passage of a few organisms through the nasal mucosa, the absorptive capabilities of which were considered to be very good. The latter assumptions have been shown elsewhere to be true (10).

Studies of pneumococcus carriers (5) and meningococcus carriers (1) in an unselected group of individuals who had had no known contact with the frank disease have shown a striking similarity to exist between the two conditions. It was therefore obviously necessary to examine the meningococcus carrier state carefully to determine whether this is to be regarded as an infection or not.

A study of coryza, pharyngitis and upper respiratory disease in a group of non-contact carriers (1) and in the carriers at Camp Rusk has failed to reveal any direct relationship between these inflammatory conditions and the duration or extent of the carrier state. Indeed, the only possible relationship, and this was little more than suggested, was that any infection accompanied by the copious outgrowth of a pathogen, such as *Hemophilus influenzae* or pneumococcus, caused the numbers of meningococci to decrease.

Attention was therefore paid to the reactions of the serum in known meningococcus carriers. Serum was obtained from 41 carriers of which 31 were Type I and 10 Type II. Serum was also obtained from 9 recovered cases, 8 of which had had intensive serum therapy

both intravenous and intrathecal. In every case the serum was tested for the presence of agglutinins and in the case of 12 Type I and 5 Type II sera and also sera from 4 recovered cases, tests were made for the presence of protective antibodies.

The agglutination tests were set up in a 37°C. water bath and then allowed to stand overnight in the ice box. The serum was tested against freshly isolated and readily agglutinable Type I and Type II strains at final serum dilutions of 1/10, 1/20 and 1/40. Of the 31 Type I sera, 21 gave no agglutination or one that was slight and equal with both Type I and Type II organisms, 6 gave moderate agglutination to 1/20 without any cross-agglutination or even slightly to 1/40

TABLE III

	Agglutination		Protection	
	Type I	Type II	Type I	Type II
Good	13, 58, 73, 95	57, 139	7, 55, 58	24, 54
Moderate	7, 45, 55, 67, 75, 167	24, 54, 141, 207	67, 73, 138, 167, 185	
None	2, 15, 16, 32, 40, 52, 66, 74, 79, 115 c, 124, 127, 132, 138, 145, 149, 152, 153, 185, 194, 198	38, 85, 115 d, 169	13, 75, 95, 194	85, 115 d, 207

and 4 gave good agglutination out to 1/40. Of the 10 Type II sera, 4 gave no agglutination or one that was slight and equal with both Type I and Type II organisms, 4 gave a moderate agglutination and 2 gave good agglutination (Table III). 21 sera from normal individuals or from persons suffering from epidemic encephalitis failed to agglutinate either Type I or Type II organisms under the conditions of the experiment.

In the protection tests use was made of the virulence test with mucin suspensions as outlined above. 0.2 cc. of the serum to be tested was injected intraperitoneally into white-face mice 30 minutes to 1 hour before inoculation of the mucin suspension of organisms. The test serum was titrated against suspensions of the organisms

ranging from 10^{-2} to 10^{-6} of the standard suspension (2,000,000,000 per cc.) using 2 mice at each dilution. Control tests were made at the same time that a batch of sera from carriers was tested and consisted of a test without any serum, one with presumably normal pooled human serum² and a third with Type I or Type II antimeningococcal horse serum of known protective potency.

Since 2 cc. of serum was the minimum required for each protection test even when tested only against organisms homologous with the type carried in the throat of the individual from whom the serum was obtained, only a limited number of the sera collected could be tested. In all, 3 experiments were made and 12 sera from Type I carriers and 5 from Type II carriers were tested. The results are shown in Table IV.

From an examination of Table IV it will be seen that 3 Type I carrier sera and 2 Type II give good protection, 5 Type I sera give a certain amount of protection as compared with the controls and 4 Type I and 3 Type II sera give no protection. In Table III these sera are shown ranged according to whether their protection was good, moderate or high, and compared with the agglutination of the same and other carrier sera also arranged in 3 somewhat arbitrary groups. It is obvious that the correspondence between content of agglutinins and content of protective antibodies as demonstrated by these tests is not close. Of the 17 sera with which both agglutination and protection tests were done, 11 showed some correspondence in containing both agglutinins and protective antibodies, while 6 showed no correspondence.

Nine sera from recovered cases were obtained. Of these only one (No. X) had not received treatment with antimeningococcal polyvalent serum. This serum gave a moderate agglutination against Type II meningococci and good protection against Type II organisms. No strain had ever been isolated from this case but the spinal fluid drawn at the height of the disease gave a moderately strong precipitin

² Owing to the limited number of white-face mice available, it was not possible to perform protection tests with large numbers of normal human sera. Under these circumstances it was decided to pool 4 normal sera and use this as a control in each protection test. Since 4 different sera were used with each test, 12 control sera in all were tested.

reaction with Type II monovalent serum. In the 8 cases in which serum therapy had been administered 1 to 4 months before blood was drawn for testing, 6 (Nos. 30, 81 *b*, 106, 154, Y, Z) showed no agglu-

TABLE IV

Type I						Type II					
Serum No.	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	Serum No.	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
{ 55	1, 1		1,			{ 24	1,				
{ 58	1,	1,	1,			{ 54	No deaths				
73	1,	2, 2	1,	2,	1, 3	{ 85	1, 1	3,			
{ 95	1, 2	2, 2	1, 1	2, 2	1, 2	{ 115 <i>d</i>	1, 1			1,	1,
{ 194	2, 2	1, 1	1, 2	1, 1	1,	{ 207	1, 1	1,			
No serum	1, 1	1, 2	2, 2	1, 2	2,	No serum	1, 2	2, 2	2,	1,	
Pooled normal serum	1, 2	1, 2	1, 1	2, 2	2, 2	Pooled normal serum	1, 2	1, 2			
Immune Type I serum	No deaths					Immune Type II serum	1,				
7	No deaths										
{ 67	1, 1	1, 1		2,							
{ 138	1,	1, 2		1,							
{ 167	1, 1	1, 1									
{ 185	1, 1	1, 1	1,								
{ 13	1, 1	1,	1, 2	1, 2							
{ 75	1, 1	1, 1	1,	2,							
No serum	1, 1	1, 1	1, 2	2,							
Normal pooled serum	1, 2	1, 2	2, 2	2,							
Immune Type I serum	No deaths										

Apr. 15, 1934. Strain 45 ² Type I	June 29, 1934. Strain 506 Type I	June 28, 1934. Strain 509 Type II
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Figures indicate duration of life in days (*i.e.*, 2 = died during 2nd day). 2 mice were tested in each dilution; blank indicates mouse survived.

tinins and 2 (Nos. 35 and V) showed good agglutinins for Type II organisms. Of these latter 2, No. 35 had received serum therapy 3½ months before being bled and the other was considerably more recent,

having received both intrathecal and intravenous serum only 3 weeks before. In the case of 3 of the 8 sera (Nos. 30, 81 *b* and Y) protection tests were made. All 3 showed moderate protection. No. 30 had received serum 4 months before, No. 81 *b* 1 month before and Y 2 months before.

It seems clear from these results on the carrier sera that there is, in a certain percentage of cases, some general reaction of the body, at least serological, to the presence of meningococci in the nasopharynx. Thus, 32.3 per cent of Type I carrier sera tested and 60 per cent of Type II sera tested showed moderate or good agglutinins for homologous organisms. Also 80 per cent of Type I sera tested and 40 per cent of Type II sera tested showed moderate or good protective antibodies against homologous organisms.

Despite the absence of any convincing local infection in meningococcus carriers, this general reaction of the body would seem to point to the fact that the carrier state is to be looked upon as an infection. It seems probable that small numbers of meningococci are continually making their way through the mucosa and that these organisms, entering the local tissues and also the blood and lymph streams, are sufficient to produce immune reactions. Such is known to be the case with experimental pneumococcus infections in animals (8, 10).

It was unfortunately quite impossible to obtain any idea in these individuals as to how long the carrier state had existed. It can only be said that for the great majority it probably was not over 9 months, the length of time the camp had been in existence, and that for many it was probably not more than $4\frac{1}{2}$ months, which was the time since the first case appeared in the camp and the time at which date the carrier rate probably first greatly exceeded 20 per cent.

SUMMARY AND CONCLUSIONS

The investigation of this isolated epidemic of meningococcus meningitis at a C.C.C. camp gave an opportunity to examine the carrier state in contacts carrying what were presumably virulent epidemic strains of organisms.

With the aid of Miller's technique for the enhancement of the demonstrable virulence of meningococci for mice, it proved possible to test the virulence of the carrier strains from Camp Rusk. These

results were consistent despite the interval of from 3 to 4 weeks which intervened between the isolation of the strains and the virulence titrations. Type I strains were found to have a high virulence, while the virulence of Type II strains was moderately high but definitely less than that of the Type I, and atypical strains and strains of *N. catarrhalis* isolated from carriers showed a very low virulence.

The question of the precise nature of the carrier state was investigated. No evidence has been obtained yet as to the existence of a relationship between pharyngitis, coryza or upper respiratory disease and the presence and degree of the carrier state. This is unlike the situation with regard to pneumococcus carriers. On the other hand, it has proved possible to demonstrate reactions within the body to the meningococci in the nasopharynx, consisting of the formation of agglutinins and protective antibodies in the blood serum. 32.3 per cent of Type I and 60 per cent of Type II carrier sera showed moderate or good agglutinins for homologous organisms and 80 per cent of Type I and 40 per cent of Type II sera showed moderate or good protective antibodies against virulent homologous strains. No idea could be obtained as to the relationship of the presence or absence and the degree of serological reaction and the duration of the carrier state.

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THE PRECIPITIN REACTION BETWEEN TYPE III PNEUMOCOCCUS POLYSACCHARIDE AND HOMOLOGOUS ANTIBODY

II. CONDITIONS FOR QUANTITATIVE PRECIPITATION OF ANTIBODY IN HORSE SERA*

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Experiments were reported several years ago by the writers (1) leading to an absolute method for the determination of precipitins (2-4). It was originally thought that by following the usual immunological technique, namely incubation of precipitin reactions at 37°C. for 2 hours and letting stand in the ice box overnight, conditions had been established for the maximum precipitation of antibody. While this has been found to be the case for rabbit antisera, experiments such as those given below have shown that the usual immunological practice does not result in maximum precipitation, or in the attainment of true equilibria, in the case of pneumococcus antisera produced in the horse.

It appears, moreover, that objections are still being made to the absolute chemical method for the estimation of precipitins on the ground that non-specific protein nitrogen might be included in the values actually found. Since non-specific protein was shown to be without influence by Marrack and Smith (5) the writers have hitherto refrained from publishing their own data on this point, but are now including such material.

EXPERIMENTAL

1. *Incompleteness of Precipitation When Carried Out Both at 37° and 0°.*—According to the usual immunological technique, precipitin tests are incubated for

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York.

2 hours at 37°C. and allowed to stand overnight in the ice box. The writers have shown that in rabbit antisera the same amount of specifically precipitable protein is thrown down whether the above conditions are employed, or whether the entire reaction is carried out at 0° (4¹). This is, however, not the case with antipneumococcus horse sera nor with antibody solutions prepared from horse sera.

Duplicate tubes were set up containing 1.0 cc. of Type III antipneumococcus horse antibody Solution B 61² and 0.05 mg. of S III in a total volume of 4.0 cc. In the first pair of tubes the reagents were chilled to 0°C. before mixing, after which the tubes were kept in the ice box overnight and whirled in the refrigerating centrifuge.³ A second pair of tubes was kept at 37° for 2 hours, allowed to stand overnight in the ice box, and was then centrifuged in the cold. A third pair of tubes was incubated for 2 hours at 37° and was then stoppered and shaken in an ice bath for 3 hours in order to favor the establishment of equilibrium. A fourth pair of tubes was placed in an incubator at 37°C. for 2 hours and whirled in a small Swedish angle centrifuge⁴ in the same incubator. The precipitates were washed twice with chilled saline (3) and analyzed for nitrogen by a modification of the micro Kjeldahl method. The results were as follows:

Tubes mixed at, °C.....	0	37	37	37
Tubes centrifuged at, °C.....	0-10	0-10	Shaken at 0	37
Antibody N pptd. by 0.05 mg. S III	1.38	1.13	1.15	1.00
	1.38	1.12	1.15	0.97
Maximum specifically pptble. N in solution (0.15 mg. S III)	2.11			1.85
	2.15			1.85

It will be seen that the largest quantity of antibody nitrogen is precipitated by a given amount of S III when the solutions are kept at low temperatures throughout. Under these conditions the precipitate forms more slowly and is less gelatinous than at 37°. Allowing the tubes to stand at 0° for an additional 24 hours does not appreciably increase the amount of antibody nitrogen precipitated. When the reaction is started at 37° and completed at 0° far less antibody nitrogen is precipitated, although the amount is greater than if the tubes are centrifuged at 37°. This appears to be due to a partial attainment of the final equilibrium arrived at when the reaction is carried out entirely at 0°, since shaking the tubes for several hours at 0° brings the amount of antibody nitrogen somewhat closer to the 0° value. Similiar results were obtained with another antibody solution, B 60, and with Type III antipneumococcus horse serum. Experiments

¹ Heidelberg, Kendall, and Soo Hoo, page 142.

² Prepared according to Felton (6).

³ Manufactured by the International Equipment Co., Boston.

⁴ Supplied by Eimer and Amend, New York.

at 37° for 4 and 24 hours showed that no more antibody was precipitated than in 2 hours.

2. *Effect of Non-Specific Protein.*—From Table I in the following paper (III) it is clear that at 0° or at 37° the ratios of nitrogen to S III in the precipitate are of the same order whether the analysis is carried out in whole serum (607), in which the antibody constitutes about 15 per cent of the total protein, or in the antibody solution (B 62) prepared from it, in which antibody is 50 to 60 per cent of the total protein. In comparing actual ratios it should be noted (see also Table VI, Paper III) that the antibody solution contains less precipitable nitrogen per cubic centimeter than the serum. This would tend to make the ratio lower at any given S level.

That the above findings are due to the actual antibody content in each instance and not to the presence of other serum components is shown in the following experiment, in which parallel determinations were made with antibody solution alone, and with antibody solution to which an equal volume of normal horse serum had been added. Reiner and Reiner (7) have shown that normal horse serum contains globulin very similar to the antibody fraction in antipneumococcus serum, so that it would be expected that a portion at least of this globulin would be carried down by S if non-specific protein were reactive.

Duplicate tubes containing 1.0 cc. of a Type I pneumococcus antibody Solution B 72 and 0.05 mg. of S I⁵ were set up at 0°, at 37°, and at 37° and 0° as described above, and the nitrogen precipitated was compared with a similar series to which 1 cc. of normal horse serum had been added. The results are given in the following tabulation and confirm those of Marrack and Smith (5) on other immune systems.

Effect of Non-Specific Protein
Antibody Nitrogen Precipitated by 0.05 Mg. S I

Temperature, °C. Centrifuged at, °C.	0 0	37 0	37 37
1.0 cc. B 72	0.92	0.57	0.51
1.0 cc. B 72 + 1 cc. normal horse serum	0.92	0.58	0.52

The above experiments show that addition of non-specific protein has no effect upon the amount of antibody nitrogen precipitated under any of the conditions of temperature investigated.

DISCUSSION AND SUMMARY

The experiments recorded above show that in the case of anti-pneumococcus horse serum or purified antibody the arbitrary im-

⁵ Experiments have shown that the reaction between Type I pneumococcus polysaccharide and its homologous antibody closely parallels that of S III.

munological procedure (37° for 2 hours, overnight in the ice box) does not permit either the establishment of a true equilibrium or the precipitation of the maximum amount of antibody nitrogen. Analyses of such horse sera for antibody content should therefore be carried out at 0° and the determinations should be allowed to stand in the cold for at least 24 hours in order to insure the completion of the reaction.

It is believed that the similarity of the nitrogen : S III ratios in the specific precipitate, whether obtained from whole serum or from purified antibody, and the failure of added serum to influence the amount of nitrogen precipitated show that the absolute chemical method for the estimation of antibody actually measures antibody and not antibody plus a more or less indefinite amount of non-specific protein. An objection to the use of the method is thus shown to be unfounded.

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THE PRECIPITIN REACTION BETWEEN TYPE III PNEUMOCOCCUS POLYSACCHARIDE AND HOMOLOGOUS ANTIBODY

III. A QUANTITATIVE STUDY AND A THEORY OF THE REACTION MECHANISM*

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In the first paper of this series (1) it was concluded as a first approximation that under a standard set of conditions the entire course of the precipitin reaction between the specific polysaccharide of Type III pneumococcus and homologous purified antibody could be quantitatively accounted for by three simple equations. The mass law was believed to hold for these equations, the more so as the reactions were found to be reversible. Studies of the theoretical factors involved have since been continued under more varied conditions, and the present paper describes experiments which have necessitated modification of the conclusions originally drawn.

EXPERIMENTAL

The quantitative precipitin determinations were made as in previous papers (2-4), except that the technique was modified as described below in order to study the effect of varying a given set of conditions. In general, precipitates were analyzed, rather than supernatants, as had been done in (1). Much of the serum used was obtained through the kindness of Dr. William H. Park, to whom the writers again wish to express their gratitude. Unless otherwise stated, antibody solutions were prepared according to Felton (6). The specific poly-

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York.

saccharide of Type III pneumococcus is referred to throughout as S III.

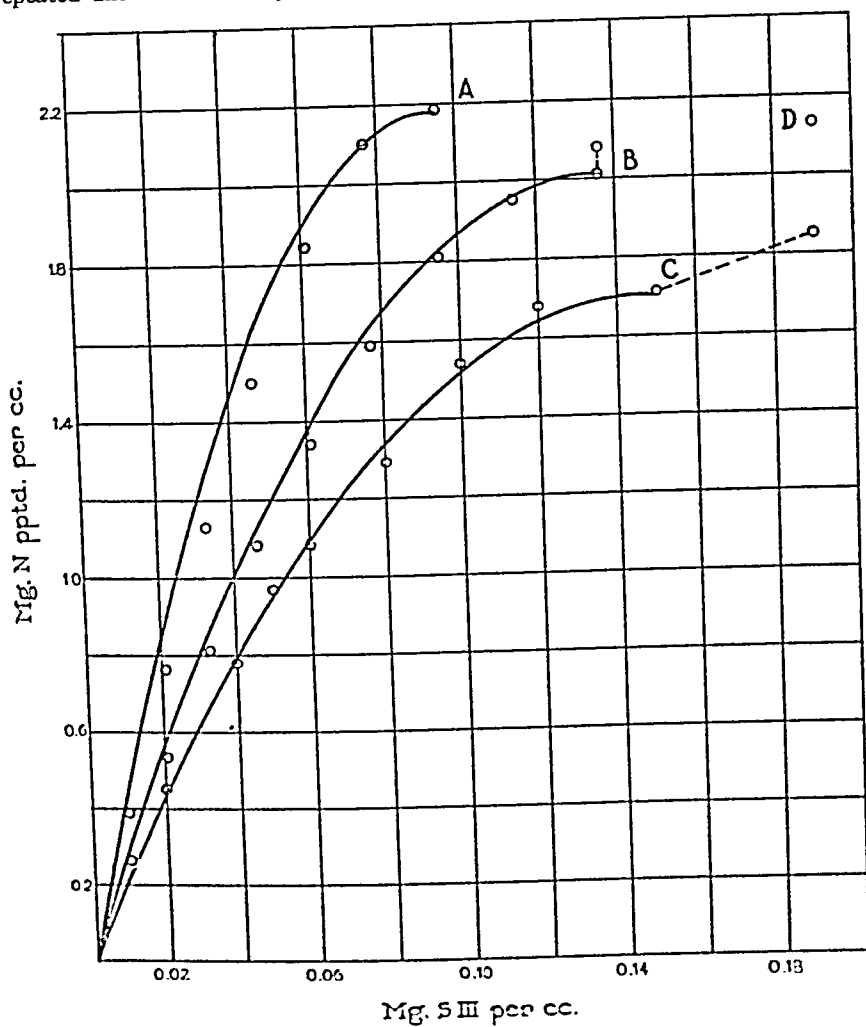
In view of the temperature effects shown in the preceding paper (7) it was found necessary to work at a single temperature in studying the reaction mechanism, and experiments were accordingly run either at 0° or at 37°C.

The differences between the amounts of antibody nitrogen precipitated when the reaction mixture is kept in the cold throughout (0°) and when the precipitation and centrifugation are carried out at 37° are strikingly shown in Tables I and II, which summarize, respectively, the results of the addition of increasing amounts of S III to 1 cc. portions of serum or antibody solution and of serial additions of small amounts of S III to antibody solutions under those conditions.

1 a. Addition of Increasing Amounts of S III to Antibody at 37° and at 0°.—Experiments corresponding to those in the earlier work (1) have now been made with the temperature constant throughout. Duplicate tubes were set up at 37° using 1.0 cc. of antibody Solution B 61 and varying amounts of S III in a total volume of 4 cc. The tubes were incubated for 2 hours at 37° and then centrifuged at that temperature. The precipitate was washed twice with cold saline and analyzed for nitrogen. The results are given in Table I, Column 2, and are represented by the circles along Curve C, Fig. 1. The point connected with the curve by the dotted line indicates the maximum amount of nitrogen specifically precipitable (0.4 mg. S III) from this solution at 37°. A similar experiment was run with antibody Solution B 62 in which the tubes were set up at 0° and centrifuged in the cold instead of at 37°. The amount of S III combined in the region of excess S III was found by determining the amount left in the supernatant by the method described in a previous paper (3), except that the determinations were run at 0° in the B 62 experiments and entirely at 37° in the B 61 series. Aliquot portions of the supernatants containing a suitable amount of S III were set up with another 1.0 cc. portion of the antibody solution used in the experiment, adding saline to bring the volume to 4 cc. The precipitates were analyzed after 24 hours. The amount of S III in the B 61 aliquots was read off from the experimental points along Curve C, Fig. 1 (Column 2, Table I). A similar curve was constructed for B 62. The results are given in Table III.

1 b. Serial Experiments at 0° and at 37°.—In these experiments successive small portions of the antibody were precipitated. In the 0° experiment the reagents were chilled in an ice bath. Duplicate 5.0 cc. portions of antibody Solution B 61 were measured into Wassermann tubes and mixed with 0.5 cc. of a 1 to 10,000 solution of S III. The tubes were kept in the ice box overnight and were then centrifuged in the cold. The supernatants from the duplicate tubes were mixed

and 5.0 cc. samples set up with 0.5 cc. of S III as before. This procedure was repeated until the antibody was exhausted. In the case of antibody Solution



TEXT-FIG. 1

B 62, 0.02 mg. portions of S III were added. The precipitates were washed and analyzed as in the preceding section.

The 37° experiment was carried out in the same way except that the solutions were mixed at room temperature. The tubes were allowed to stand for 2 hours at 37° and were then centrifuged in a small angle centrifuge at 37°.

TABLE I
Addition of Increasing Amounts of S III to 1.0 Cc. of Serum or Antibody

Antigen S III added	Antibody Solution B 61, 37°				Horse Serum 607, 0°				Antibody Solution B 62, 0°				Horse Serum 607, 37°				Antibody Solution B 62, 37°			
	N pptd.		N:S III in ppt.		N pptd.		N:S III in ppt.		N pptd.		N:S III in ppt.		N pptd.		N:S III in ppt.		N pptd.		N:S III in ppt.	
	mg.			Tests on supernatant	mg.			Tests on supernatant	mg.			Tests on supernatant	mg.			Tests on supernatant	mg.			Tests on supernatant
0.01	0.45	22.5	No S, xs A*		0.62	31.0	No S, xs A		0.36	36.0		No S, xs A	0.42	21.0		No S, xs A	0.44	22.0		No S, xs A
0.02									0.57	28.5		" " "	0.74	18.5		" " "				
0.03	0.79	19.8	" " "		1.03	25.8	" " "		0.78	26.0		" " "				" " "				
0.04	0.97	19.4	" " "						1.07	21.4		" " "	1.16	15.5		" " "	0.96	16.0		" " "
0.05	1.08	18.0	" " "		1.25	20.8	" " "					" " "	1.23	13.7		" " "				
0.06					1.35	18.0	" " "		1.25	16.7		No S or A				" " "				
0.075	1.29	16.1	" " "									" " "	1.35†	9.0		No S or A	1.18†			Excess S
0.08					1.40	15.6	" " "		1.29	12.9		" " "	1.32†			Excess S	1.20†			" "
0.09	1.54	15.4	" " "		1.43†	14.3	No S or A					" " "				" " "				S, trace A
0.10	1.68	14.0	" " "		1.51†	12.6	" " "		1.25			" " "				" " "				
0.12	1.71	11.4	" " "		1.59†	10.6	" " "					" " "				" " "				
0.15												Excess S								
0.20	1.75		Excess S																	
0.25					1.45															

* S = S III; A = antibody; xs A = excess antibody.

† Not run in duplicate.

TABLE II
Serial Additions of S III to Antibody Solutions B 61 and B 62

Precipitation No.	B 61, 0.05 mg. S III used				B 62, 0.02 mg. S III used	
	37°		0°		0°	
	Antibody N pptd.	Ratio N : S III in ppt.	Antibody N pptd.	Ratio N : S III in ppt.	Antibody N pptd.	Ratio N : S III in ppt.
	mg.		mg.		mg.	
1	1.32	26.4	1.94	38.8	0.83*	41.5
2	1.24	24.8	1.70*	34.0	0.82†	41.0
3	1.15	23.0	1.53	30.6	0.72	36.0
4	1.01	20.2	1.39	27.8	0.61	30.5
5	0.88	17.6	1.17‡		0.56	28.0
6	0.77	15.4	0.80‡		0.47	23.5
7	0.63‡		0.23‡		0.43‡	
8	0.37‡		No ppt.		0.21‡	
9	0.14‡				No ppt.	
10	0.15§					

* One determination lost.

† One determination discarded.

‡ The supernatants from these precipitates gave tests for the presence of S III with excess antibody.

§ 5 cc. supernatant from tube 9, allowed to stand in the ice box overnight, gave this additional precipitate. After centrifugation the supernatant showed a negative reaction for antibody with S III.

TABLE III
Determination of N:S III Ratio in Presence of Excess S III

Antibody B 62, set up at 0°, centrifuged at 0-10°							
Amount S III	Antibody N pptd.	Fraction of supernatant used for determination of S III content	Antibody N pptd.	S III in aliquot	Total S III in supernatant	S III in original ppt. (by difference)	N : S III in original ppt.
mg.	mg.		mg.	mg.	mg.	mg.	
0.50	1.25	1/8	0.74	0.029	0.23	0.27	4.6
1.00	1.19	3/50	1.01	0.044	0.73	0.27	4.4
1.50	1.18	1/20	1.17*	0.059	1.18	0.32	3.7
2.00	1.16	1/40	1.00*	0.044	1.76	0.24	4.8
3.00	1.02	1/50	1.11	0.053	2.65	0.35	2.9
Antibody B 61, set up at 37°, centrifuged at 37°							
0.25	1.75	3/4	0.17	0.007	0.009	0.241	7.3
0.30	1.79	3/4	0.30	0.013	0.017	0.283	6.3
0.35	1.83	3/4	0.48	0.022	0.029	0.321	5.7
0.40	1.85	1/2	0.56	0.027	0.054	0.346	5.3
0.50	1.84	1/4	0.54	0.025	0.100	0.400	4.6
0.75	1.76	1/8	0.89	0.046	0.368	0.382	4.6
1.00	1.76	3/40	0.92	0.048	0.640	0.36	4.9
1.50	1.65	1/20	1.02	0.059	1.15	0.32	5.2

* One analysis discarded.

QUANTITATIVE PRECIPITIN STUDY

The values given in the tables represent the mean of the duplicate determinations unless otherwise stated.

The results of the 0° and 37° serial experiments with B 61 are represented by circles in Fig. 1 along Curves A and B, respectively, calculating each point back 1.0 cc. volume. The method of calculating the smooth Curves A, B, and C given in the theoretical part. The point connected with B by the dotted line indicates the additional amount of antibody nitrogen precipitated when the

TABLE IV
Effect of Volume, Final Concentration of Antibody, and Time of Standing

Volume cc.	Antibody B 62 at 0°C.				Antibody B 61 at 37°C.			
	Antibody N pptd. by 0.03 mg. S III in 24 hrs.	Concen- tration antibody N	Antibody N pptd. by 0.03 mg. S III in 48 hrs.	Concen- tration antibody N	Antibody N pptd. by 0.05 mg. S III	Concen- tration antibody N	Antibody N pptd. by 0.10 mg. S III	Concen- tration antibody N
	mg.	mg. per cc.	mg.	mg. per cc.	mg.	mg. per cc.	mg.	mg. per cc.
2	0.88	0.21	0.87	0.21	0.87	0.25	1.15	0.18
4	0.84	0.11	0.91	0.10	0.87	0.12	1.16	0.09
6	0.83	0.08	0.87	0.07	0.87	0.08	1.16	0.06
8	0.88	0.05	0.84	0.06				
10	0.84	0.05	0.84	0.05				
12	0.82	0.04	0.87	0.04	0.85			

TABLE V
Nitrogen Precipitated from Antibody B 62 by Methylated S III at 0°

Methylated S III mg.	N pptd. mg.	Supernatant tested with		
		Antibody	Methylated S III	S III
0.05	0.43	—	++	++
0.10	0.60	+	+	++
0.15	0.68	±	±	
0.25	0.74	++	—	
0.50	0.83	++		

solution, exhausted at 37°, was cooled to 0°. Point D represents the maximum amount of nitrogen specifically precipitable from 1.0 cc. of B 61 (0.5 mg. S III) at 0°.

2. *Effect of Changes in Volume and Concentration.*—Duplicate portions of 1.0 cc. of antibody Solution B 62 were set up at 0° with 0.03 mg. of S III in volumes of 2, 4, 6, 8, 10, and 12 cc. One set of tubes was allowed to stand for 24 hours, the other for 48 hours in the ice box. The precipitates were centrifuged off in the cold and aliquot portions of the supernatant were analyzed. Duplicate portions of 1.0 cc. of B 61 were also set up with 0.05 and 0.10 mg. of S III, and after 2 hours at

37° the tubes were centrifuged at room temperature. The results are shown in Table IV.

3. *Reaction of Pneumococcus III Antibody with Methylated S III.*—S III was methylated with methyl sulfate and sodium hydroxide.¹ Solutions of the sodium salt of the methylated product were found to precipitate with homologous horse antipneumococcus serum but not with the corresponding rabbit serum. The failure to precipitate the Type III rabbit antiserum shows that the reaction in horse serum is not due to small amounts of unchanged S III, the more so because addition of traces of unmethylated S III to the methyl S III results in a prompt reaction with rabbit antiserum. Table V shows that the methylated product precipitates 0.83 mg. of nitrogen from 1.0 cc. of antibody Solution B 62 which contains 1.25 mg. of nitrogen precipitable with S III, or 65 per cent of the total antibody.

An antibody solution was made by Felton's method from serum which had been completely absorbed with the methylated S III. This solution contained 1.60 mg. per cc. of antibody nitrogen precipitable by S III and gave no reaction with the methylated product. It also reacted strongly with the partially hydrolyzed S III fractions which have been described in an earlier paper (8) and which did not precipitate with rabbit antisera. It is believed that these findings necessitate the conclusion that more than one antibody to the carbohydrate is present in the original serum or antibody solution.

DISCUSSION

As a result of a new study of the precipitin reaction under definite temperature conditions in the region of excess antibody ratios far higher than in Reference 1 have been found for antibody nitrogen to S III in the precipitate, ranging as high as 40:1, or greater, at 0° and in the presence of a large excess of antibody. The equivalence point² ratios, however, remain much the same, averaging 10.8:1.

In a previous paper (1) it was shown that between the equivalence point and the beginning of the inhibition zone there was a wide range in which additional S III caused no change in the amount of nitrogen precipitated. This range, in which S III is present in excess, has now been investigated in greater detail. It has been found that when increasing amounts of S III are added to a fixed quantity of antibody the amount of S III in the precipitate increases to a maximum and then remains almost constant until solution of the precipitate begins. The limiting value of the N : S III ratio in this zone is very close to 5 and is not affected by temperature changes (Table III).

¹ Details of the methylation will be given in a later paper.

² Designated equilibrium point in Reference 1.

QUANTITATIVE PRECIPITIN STUDY

Thus the ratio of antibody nitrogen to S III varies between more than 40:1 and about 5:1 in the reaction range in which a precipitate is formed. It was also shown in the earlier studies (1) that the soluble compound formed in the inhibition zone contains one more molecule of S III than the immediately preceding insoluble compound. This great variation in composition indicates that antibody and S III are multivalent with respect to each other ((1); also (5, 9, 10), Arhenius (11), Fleischmann and Michaelis (12), for data on other systems)). The chemical structures of both components of the reaction afford strong support for this view. S III is a polysaccharide built up of at least 10 identical aldobionic acid units joined by glucosidic linkages (13). The group or groups responsible for its reactivity must thus be repeated many times in the molecule. The fact that the partial hydrolysis products react with antibody (8) shows that the reaction is due to certain groupings in the molecule and is not a property of the molecule as a whole. The antibody appears to be a serum globulin of higher molecular weight than normal serum globulin.³ Since it is built up of amino acid units it, too, offers opportunity for the repetition of the groups involved in the precipitin reaction.

The experiment on the effect of volume upon the amount of antibody precipitated (Table IV) shows that changes in the concentration of antibody in the supernatant have no effect upon the ratio of antibody nitrogen to S III in the precipitate. This apparent contradiction to the requirements of the mass law shows that the explanation of the reaction given in Reference 1 is not adequate. However, the difficulty raised by this finding might with equal justice be urged against attempts such as those of Burnet (14) and Taylor (15) to explain the varying ratios on the basis of adsorption. Thus the Freundlich adsorption equation $\frac{y}{a} = Kx^{\frac{1}{n}}$ states that the amount adsorbed per unit of surface is proportional to some power of the concentration. If, however, one accepts the writers' conclusions, expounded below, regarding the nature of the reaction, one may still use simple chemical equations and apply the mass law, as will be seen in the mathematical treatment in the following section.

One reason for the selection of the S III-antibody system for study

³ Unpublished diffusion experiments.

was the fact that the S III could be prepared in a pure state. It was felt that one of the components of the reaction was a single substance, and that for this reason the homologous antibody might also be homogeneous. However, evidence has been accumulating which indicates that different parts of a hapten molecule may act independently in stimulating the formation of antibodies and in reacting with them. The cross-reactions between the antibodies formed in response to injection of proteins linked to certain haptens which have been studied extensively by Landsteiner (16) and by Avery, Goebel, and Babers (17) lead to this conclusion (*cf.* also Hooker and Boyd (18)). This is now shown to apply to S III as well. If the hydroxyl groups are covered by methyl groups, leaving the carboxyl groups free, the resulting compound is still reactive with Type III pneumococcus antcarbohydrate. However, it then precipitates only two-thirds of the antibody present (see Table V). Dissociation of the antibody-methyl S III compound does not explain this finding, for the supernatant, after purification and concentration by the Felton method, still failed to precipitate methyl S III. The remainder of the antibody may be precipitated by unmethylated S III, in which the hydroxyl groups are free to react. This indicates not only that more than one kind of antibody is present, but also that on the S III molecule there is, in addition to the molecular grouping carrying hydroxyl groups, at least one other molecular grouping which is immunologically reactive and independently so.

Other observations also show that the antibody is a mixture. In serial experiments after much of the antibody has been removed by successive additions of S III, a point is reached at which an appreciable quantity of S III occurs in the supernatant in the presence of a concentration of antibody which would precipitate S III in a dilution of 1:10,000,000 if the antibody remaining had the same properties as the original antibody. Thus the last portions of antibody to be precipitated appear to show a higher dissociation in the reaction with S III than do the portions which react first.

Again, the difference in the quantity of antibody precipitated at 0° and 37° indicates that a fraction of the antibody forms such soluble or highly dissociated compounds with S III that they cannot be completely precipitated at the higher temperature. The fact that the same amount of antibody is precipitated by S III at 37° from volumes of 4, 8, and 12 cc. (Table IV) shows that the difference is not due to different solubilities of a homogeneous antibody-S III complex at the two

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temperatures or to dissociation of such a complex. There is a difference of 0.20 mg. in the amount of nitrogen precipitated when 0.05 mg. of S III and 1.0 cc. of B 61 are set up in a volume of 4 cc. at the two temperatures. The difference should be twice as great in a volume of 8 cc. and three times as great in a volume of 12 cc. if solubility of the precipitate were the cause of this discrepancy, and should be even greater if dissociation of a single compound occurred as well. Thus the serial experiments, the result of carrying out the precipitin reaction at different temperatures, and the experiments with methylated S III all indicate that when horses are immunized with Type III pneumococcus more than one antibody is formed which is reactive with the homologous specific polysaccharide.

SUMMARY

1. In the precipitin reaction between the specific polysaccharide of Type III pneumococcus (S III) and homologous antiserum or purified antibody derived from the horse, the temperature at which the reaction is carried out influences the amount of antibody precipitated.
2. The course of the S III-antibody reaction was studied both at 37° and at 0° from the region of excess antibody to the region of excess hapten. Over the whole range the ratios of antibody nitrogen to S III in the precipitate varied from more than 40:1 to less than 5:1.
3. The amount of antibody nitrogen precipitated under a given set of conditions was found to be uninfluenced by the actual antibody concentration, but to depend on the relative proportions of S III and antibody.
4. This and other evidence is considered to indicate the presence in the antibody solutions and sera of more than one antibody reactive with S III.
5. The significance of the findings is discussed in terms of the multivalence of S III and homologous antibody with respect to each other.

THEORETICAL PART

Although the principal conclusions arrived at in an earlier paper (1) on the mechanism of the precipitin reaction between the specific polysaccharide of Type III pneumococcus (S III) and homologous antibody still appear valid, it has been found that variation of the experimental conditions produces changes of such character that the earlier formulation no longer appears adequate. It is nevertheless possible,

starting from the laws of classical chemistry, to propose a mechanism for the S III-antibody reaction which accounts for the findings, including the Danysz phenomenon. With certain simplifying assumptions this theory permits the formulation of mathematical expressions which quite accurately describe the experimental results and are applicable to unknown sera. A similar mechanism accounts quantitatively for other instances of hapten-antibody and antigen-antibody interaction, as will be shown in subsequent papers.

In the discussion which follows, antibody is considered to be a protein which may be accurately estimated through the determination of nitrogen in the washed specific precipitate (2-4, 7). Pneumococcus antcarbohydrate occurs in the water-insoluble globulin fraction of antipneumococcus horse serum (6) and must be redissolved in the presence of salt. It exists in solution as a globulin-salt complex (for example, Pauli (19)) and it is this complex which is called antibody (A). It is also considered that S III is a definite chemical compound in a state of high purity (13), so that when the extremely delicate test with homologous antibody fails to reveal its presence in the liquid over a specific precipitate it may be assumed that the entire amount added is in the precipitate.

If increasing, small quantities of S III are added to an excess of antibody, decreasing amounts of antibody are found in the supernatant from the specific precipitate, and a point is finally reached at which "equivalent" amounts of S III and antibody are present and only minimal amounts, if any, both of antibody and S III, may be detected in the supernatant. The writers have termed this stage of the precipitin reaction the "equivalence point" (20), and since it is of importance in the discussion which follows and has been made use of in other connections (21) a detailed consideration of the concept is now given.

The location of the equivalence point with any degree of exactness presents both theoretical and experimental difficulties. If increasing amounts of S III are added to antibody a point is eventually reached at which only traces of antibody remain. This would be the actual equivalence point, were it not for at least two factors. One of these is the dissociation of the antibody-S III compound, which varies with the temperature at which the precipitin reaction is carried out and with the individual serum used. A second factor is the ability of the anti-

body-S III compound to combine with more S III in the region of the equivalence point. As the result, when the amount of S III is increased beyond the point at which traces of antibody are still present in the supernatant, a zone ensues in which small amounts of A and S III are present simultaneously, or in which tests for both A and S III are negative. This might be termed the "equivalence zone." It is followed, as the amount of S III is still further increased, by the appearance of S III in the supernatant in excess. This could be taken to mark the end of the equivalence zone, or, if the reaction were being studied in the inverse sense, by addition of increasing amounts of antibody to relatively much S, it would mark the beginning of the equivalence zone from the side of excess S. The midpoint of the equivalence zone would be the actual equivalence point, as nearly as it could be determined.

The extent of the equivalence zone depends on the individual serum studied, and for a given serum, on the experimental conditions used. It also varies with the hapten-antibody or antigen-antibody system studied.

In Column 2 of Table VI are given approximate N : S III ratios at the beginning of the equivalence zone. These were used in making the calculations in Tables VII, VIII, and IX, since the equivalence zone was approached from the region of excess antibody. In Column 4 are given the approximate ratios at the end of the zone, while in the last column are given the mean ratios, or equivalence point ratios. In Fig. 2 is given a graphic representation of the equivalence zone and the reaction on both sides of the zone,⁴ taken from data in Tables I and III for antibody Solution B 62 at 0° (Curve A, Point E on Curve C), and B 61 at 37° (Curves B and C). Point D should be at S III = 3.68.

From Table VI it is apparent that the differences in the equivalence points of the individual antibody solutions lie outside even the large experimental error involved in their determination, and this is characteristic of other immune systems as well. It is, therefore, scarcely

⁴ The breadth of the zone in some instances may explain the failure of the "optimal proportions" method to yield the same end-point when antigen is diluted as when antibody is diluted, since the equivalence zone would be approached from a different side in each instance.

possible as was formerly thought, to consider each hapten-antibody, or antigen-antibody system as characterized by a definite equivalence point, although a fairly characteristic average may be found for each system.

Another basis for the discussion which follows is the finding that the ratio of antibody nitrogen to S III in the precipitate depends on the relative proportions of S and A present, and not on their final concentrations. The difficulties raised by the finding that the antibody is a mixture of substances with different reactivities toward S III are

TABLE VI
N:S III Ratios of Various Antibody Solutions

Serum or antibody solution	Ratio at beginning of equivalence zone	Calc. ratio at beginning of equivalence zone	Ratio at end of equivalence zone	Mean ratio (equivalence point)
B III		11.5		11.1
B IV		7.9		8.7
B V _A	13.6	10.5	8.6	(11.1)
B VII		10.8		12.2
B X	(15)	12.5	11.9	(13.5)
B 32				8.7
B 36	12.4	12.4	9.4	(10.9)
B 51	14.1	15.5	(7)	(10.6)
B 60	13.7	13.8	8.4	10.5
B 61, 37°	11.4	11.4	8.8	(10.1)
B 62, 0°	(17)	16.6	8.3	(12.7)
B 62, 37°	12	12.5	7.9	(10)
Serum 607, 0°	(15)	15.3	(8)	(11.5)
Serum 607, 37°	(11)	11.3	(8)	(9.5)
Mean equivalence point ratio.....				10.8

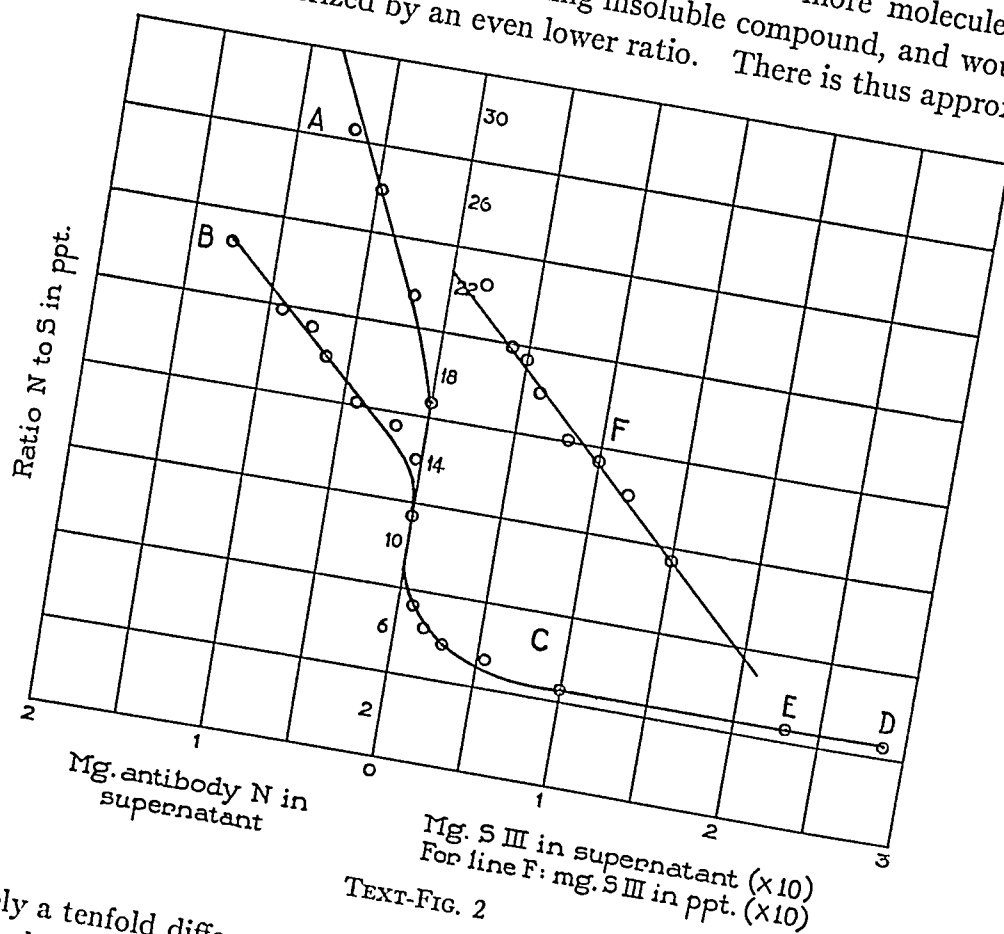
Values in parentheses indicate most probable value deduced from nearest actual determination.

met by assuming that the average behavior of the antibody is that of a single substance; that is, that it behaves statistically as if it were homogeneous. This necessarily results in a more or less artificial structure, but it appears to fit the facts and to be applicable to antigen-antibody systems in general. It is, therefore, offered as an expedient until such time as it may be possible to separate from the complex antibody mixture an antibody possessed of a single chemical reactivity.

Moreover, in the discussion which follows, the multivalence of S III and antibody with respect to each other is an essential premise. It

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was shown in the preceding sections that ratios of precipitated antibody nitrogen to S III in the region of excess antibody might be greater than 40:1, while in the region of excess S III the ratio might be less than 5:1. It was also shown previously (1) that the soluble compound formed in the inhibition zone contains one more molecule of S III than the immediately preceding insoluble compound, and would thus be characterized by an even lower ratio. There is thus approxi-



mately a tenfold difference in the extremes of the ratios in which S III and antibody may combine with each other.

A simple explanation of these varying ratios could be based on the observations that more than one antibody and more than one reactive grouping on the S III molecule are involved in the reaction. Thus S III might be considered as having Groups *a*, *b*, *c*, and *d* which react with the corresponding Antibodies *a'*, *b'*, *c'*, and *d'*. If the antibodies are present in different proportions the first compound might be S-*a'*, *b'*, *c'*, *d'*; then after *d'*, the antibody present in smallest amount, is used up,

the precipitate formed would be $S\text{-}a'$, b' , c' , and later $S\text{-}a'$, b' and $S\text{-}a'$ would be formed. This would account for the changing ratios but it does not explain the Danysz phenomenon, since the same amount of antibody would be precipitated by a given amount of S III regardless of whether all of the S III were added in one portion or in several. That this is not the case is illustrated in Fig. 1, in which Curve C shows the amount of antibody nitrogen precipitated from 1.0 cc. of antibody solution by the addition of various quantities of S III, while Curve B shows the amount of nitrogen precipitated in a serial experiment by the same quantities of S III.⁵ It will be seen that all of the antibody is precipitated by a smaller amount of S III in the serial experiment than in the other.

In order to explain the varying ratio of antibody nitrogen to S III in the precipitate, as well as the Danysz phenomenon,⁶ it is postulated that at the equivalence point the compound AS (or, more exactly, A_xS_y) is formed. In the region of excess antibody A_2S , A_3S , A_4S , A_mS (or, more exactly, $A_{mx}S_y$) may exist, depending on the relative excess of antibody. With excess S III, AS_2 can be formed as an insoluble compound and AS_3 (or, more exactly, A_xS_{3y}) as a soluble compound.

The application of the mass law to this reaction presents difficulties. If the compounds formed are considered to be A_4S , A_3S , A_2S , and AS (at the equivalence point), then as increasing amounts of S III are added to a fixed amount of antibody solution, the compound A_4S should be formed until the concentration of A is reduced to a point at which A_3S would begin to form. Throughout this range the ratio would be constant. Then on addition of more S III all of the precipitate would be converted into A_3S before any change in antibody concentration would take place. In this range the ratio would change, but the amount of antibody nitrogen precipitated would remain constant. Thus the reaction would proceed in a series of steps instead of in the continuous curve shown in Fig. 1. However, if the compounds formed are $A_{4x}S_y$, $A_{3x}S_y$, etc., intermediate steps $A_{4x-1}S_y$, $A_{4x-2}S_y$, $A_{3x}S_y$ could occur and the steps be brought so close together that a continuous curve would result. In any case, the particular compound formed would be fixed by the concentration of antibody in the supernatant.

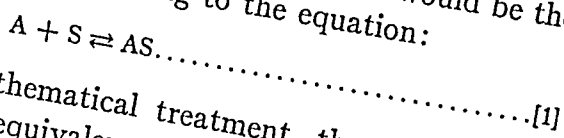
However, it has been shown that changes in the concentration of antibody in the supernatant have no effect on the ratio of antibody nitrogen to S III in the precipitate. If the mass law is to be applied it is therefore necessary to find some other basis for its use. Such a

⁵ This curve was obtained by calculating each stage back to 1.0 cc. of antibody.

⁶ Danysz himself accounted for the effect which he discovered on the basis of the union of antigen and antibody in more than one proportion (22), but his explanation was not acceptable at the time (cf. 20).

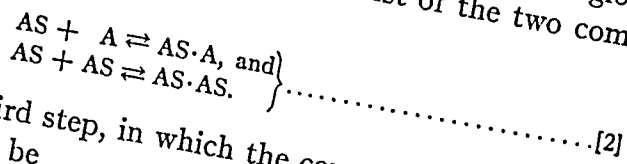
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basis is found if the S III-antibody reaction is considered as a series of successive bimolecular reactions which take place before precipitation occurs. The assumption that the reactions are bimolecular appears reasonable, for studies in all fields of chemistry have shown that more complex reactions are extremely rare. On this basis, then, the first step in the reaction between A and S III would be the formation of the compound AS according to the equation:

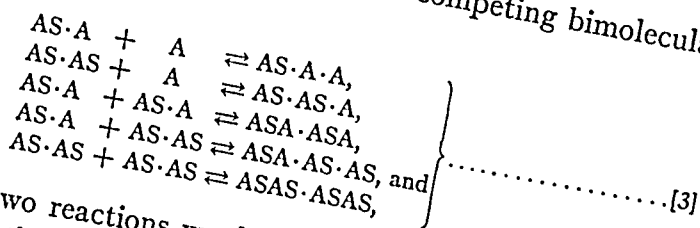


In order to simplify the mathematical treatment, the subscripts x and y are dropped and the equivalence point compound is assumed to be AS. That this procedure has only a small influence on the final result is shown later. As both S III and antibody are multivalent with respect to each other the AS compound formed in this reaction could react with other molecules of the same compound, or with S III or A, whichever is present in excess.

Let us consider the subsequent course of the reaction in the region of excess antibody. The second step would consist of the two competing bimolecular reactions:



There would follow a third step, in which the competing bimolecular reactions involved would be



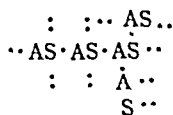
in which the first two reactions would occur only in the presence of enough A to carry the composition of the reaction product beyond the A_2S stage. Similarly, each compound formed in the third step would react with each other compound, or with more A, if present, to form still more complex substances, and the reaction would continue until particles would be formed large enough to settle from the solution and precipitation would take place.⁷

⁷ Specific precipitates have been included among the "symplexes" (Willstätter, R., and Rohdewald, M., *Z. Physiol. Chem.*, 1934, 225, 103).

If A and S III are mixed in equivalent proportions the AS formed in reaction [1] would merely polymerize in steps [2], [3],, and the equivalence point precipitate would be $(AS)_n$.

In the region of excess S III a similar series of expressions would apply, in which S and A would be interchanged in [2], [3], In the presence of a large excess of S, in other words, in the inhibition zone, there would also be present in solution a soluble compound, AS_2 , containing one more molecule of S in combination than the last insoluble compound (1). Since this is formed only with a very large excess of S, all of the specific groupings of A would tend to react with S rather than with AS complexes and there would be no large, insoluble, intermolecular aggregates formed.

The final precipitate, then, would in each case consist of antibody molecules held together by S III molecules,



a view similar to that presented recently by Marrack (23) but, it is believed, more definite and more easily treated quantitatively. The process of aggregation as well as the initial hapten-antibody combination is considered to be a chemical reaction between definite molecular groupings. On this basis it is unnecessary to make assumptions as to the change of so called hydrophilic groups into hydrophobic^a groups, as the process of aggregation would occur regardless of the affinity of the groupings for water.

It is believed that the compounds formed in the first stages of the reaction are soluble. Indeed, in the reaction between antibody and a hapten (H) containing only one reactive grouping, compounds of the type AH , would be the only ones formed and there would be no opportunity for the building up of aggregates large enough to precipitate. However, with the specific grouping repeated two or more times, as in azo dyes studied by Landsteiner and van der Scheer

^a The combination of the polysaccharide S III with the antibody would increase the number of "hydrophilic groups" on the molecule rather than decrease them, so that Eagle's explanation for the insolubility of the precipitate would not be applicable (24).

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(25), compounds of the type $AH \cdot AH \dots$ could be formed and the precipitation actually observed is accounted for. Marrack and Smith have already suggested the necessity of the presence of more than one specific grouping in a hapten in order that specific precipitation may occur (26, 23).

The mathematical treatment of the entire course of the reaction involves certain simplifying assumptions, some of which have already been discussed. It is assumed, first, that the antibody mixture may be treated statistically as though it were homogeneous; second, that in the initial stage of the reaction A reacts with S to give only AS ; third, that in the second stage of the reaction the products are $AS \cdot A$ and $AS \cdot AS$; fourth, that the mass law applies, so that the rates of formation of $AS \cdot A$ and $AS \cdot AS$ are proportional to the concentrations of the reacting substances; and fifth, that the dissociation of $AS \cdot A$ and $AS \cdot AS$ is negligible. Although there is no reason to assume discontinuities in the building up of the final aggregates, the reactions are arbitrarily treated as successive stages in order to simplify the mathematics involved.

At the beginning of the second stage of the reaction, then, in the presence of excess antibody,

let

A = total units of antibody in the reacting system,

B = units of AS formed in the first step = units of S added,

$A - B$ = units of free antibody at end of first step,

x = units of $AS \cdot A$ formed at time t ,

y = units of $AS \cdot AS$ formed at time t , and

V = volume.

Then $\frac{A - B - x}{V}$ = concentration of free antibody at time t , and

$\frac{B - x - 2y}{V}$ = concentration of AS at time t .

Rate of formation of $AS \cdot A = \frac{dx}{dt} = K \left(\frac{A - B - x}{V} \right) \left(\frac{B - x - 2y}{V} \right) \dots \dots \dots [4]$

Rate of formation of $AS \cdot AS = \frac{dy}{dt} = K' \left(\frac{B - x - 2y}{V} \right)^2 \dots \dots \dots [5]$

Dividing [4] by [5], $\frac{dx}{dy} = \frac{A - B - x}{B - x - 2y}$ if $K = K'$.

Integrating, $\frac{A - 2x}{2(A - B - x)^2} = \frac{y}{(A - B - x)^2} + C$

To evaluate C : at start of reaction when $t = 0$, $x = 0$, $y = 0$, $C = \frac{A}{2(A - B)^2}$

At the end of the reaction $x + 2y = B$. Therefore,

$$x = \frac{AB - B^2}{A} \text{ and } y = \frac{B^2}{2A}$$

Since each unit of x and y contains 2 units of antibody the number of units of antibody precipitated is given by $2(x + y)$ which equals

$$2B - \frac{B^2}{A} \dots \dots \dots [6]$$

It will be noted that the volume factors cancel, so that the amount of antibody precipitated depends only on the relative amounts of antibody and S III present and not on their concentration.

This treatment of the problem involves only the formation of compounds having ratios between R and $2R$, where R is the ratio of antibody to S III in the equivalence point compound. The experimental data show that compounds having ratios greater than $2R$ may be formed, for at 0° in the presence of a large excess of antibody ratios greater than $4R$ are encountered. By extending the process used for the calculation of the second step to stage [3] and beyond, it is possible to calculate the amount of antibody precipitated by a given amount of S III when the ratio varies between R and $3R$ and also between R and $4R$.

The calculations are complicated, as step [3] involves the bimolecular formation of five compounds, that is, A_2S , A_3S_2 , A_4S_2 , A_4S_3 , and A_4S_4 , and extension of the process to $4R$ results in 20 compounds. In this calculation it is assumed that the ratio in which any two products are formed is unaffected by the other competing reactions. The expression thus calculated for antibody precipitated in the range R to $3R$ is:

$$\text{Units of antibody pptd.} = A - \frac{2(A - B)^4}{A \{ (A - B)^2 + A^2 \}} \dots \dots \dots [7]$$

* Copies of the derivation will be furnished on request.

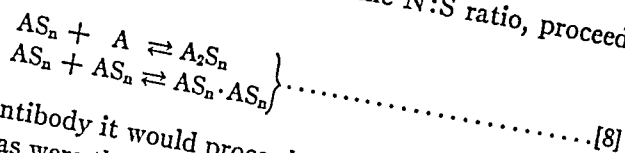
The same formulas apply in the region of excess S, and in their derivation S and A are interchanged.

In the above calculations the simplifying assumption was made that the composition of the precipitate at the equivalence point is represented by the molecular formula AS . It will now be shown that this assumption is not necessary, and that if the antibody nitrogen: S III ratio in the precipitate varies between the value found at the equivalence point and one twice as great when a large excess of antibody is present, the reaction follows the same course regardless of the molecular composition at the equivalence point.

If the compound at this point be taken as AS_n , formed as a result of a series of bimolecular reactions between A and S, making up the first step of the reaction, the

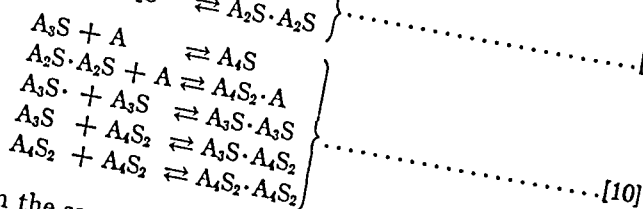
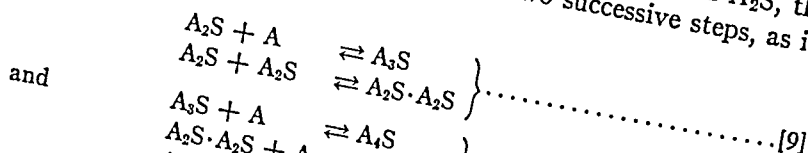
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course of the reaction as far as A_2S_n , which has double the N:S ratio, proceeds similarly to [2]:



In the presence of still more antibody it would proceed according to [3], and both reactions would be calculated as were these steps.

If, on the other hand, the equivalence point compound be taken as A_2S , the course of the reaction between A_2S and A_4S requires two successive steps, as in [2] and [3]:



and is therefore calculated in the same way as the reaction between AS and A_3S . The expression derived for the reaction between the limits A_2S and A_4S is:

$$\text{Units antibody N pptd.} = A - \frac{2(A - 2B)^4}{(A - B)[(A - B)^2 + (A - 2B)^2]} * \dots\dots [11]$$

* Copies of the derivation will be furnished on request. In Table VII, Columns 1, 2, and 3, a calculation of the reaction is given according to [1] and [2], and [9] and [10], respectively, and it is evident that the differences are small.

In making this calculation and in applying the derived equations to the experimental data it is necessary to convert units of antibody and S III into milligrams. This may be done by assuming that 1 mg. of antibody nitrogen equals 1 unit, that the number of milligrams of antibody nitrogen precipitated at the equivalence point equals A, and that the ratio of A to S III at this point is equal to R. It follows that in equations [6] and [7] $B = A$ and $B = RS$ at the equivalence point. Equation [6] then becomes:

$$\text{mg. antibody N pptd.} = 2RS - \frac{R^2S^2}{A} \dots\dots\dots [6a]$$

Equation [7] becomes

$$\text{mg. antibody N pptd.} = A - \frac{2(A - RS)^4}{A[(A - RS)^2 + A^2]} \dots\dots\dots [7a].$$

and since in equation [11] $2 B = A$ at the equivalence point and $2 B = RS$, this equation becomes:

$$\text{mg. antibody N pptd.} = A - \frac{2(A - RS)^4}{\left(A - \frac{RS}{2}\right) \left[\left(A - \frac{RS}{2}\right)^2 + (A - RS)^2\right]} \dots [11a]$$

In order to permit comparisons to be made between antibody solutions containing different amounts of antibody and having different equivalence point ratios it was found convenient to reduce the amounts of S III and N precipitated to percentages of the quantities precipitated at the equivalence point. To convert [6 a] into an expression involving percentages, use is made of the relationship $A = RS_{\text{eq.}}$ at the equivalence point. Dividing all terms of [6 a] by $RS_{\text{eq.}}$

$$\frac{N \text{ pptd.}}{RS_{\text{eq.}}} = 2 \frac{RS}{RS_{\text{eq.}}} - \frac{\frac{(RS)^2}{RS_{\text{eq.}}^2}}{\frac{A}{RS_{\text{eq.}}}} = \frac{N \text{ pptd.}}{A} = 2 \frac{S}{S_{\text{eq.}}} - \frac{\frac{S^2}{S_{\text{eq.}}^2}}{\frac{A}{A}}$$

Multiplying each side of the equation by 100,

$$\text{Per cent A pptd.} = 2 \times \%S - \frac{\%S^2}{100} \dots \dots \dots [6b]$$

$$\text{Per cent A pptd.} = 100 - \frac{2(100 - \%S)^4}{100 [(100 - \%S)^2 + 100]} \dots \dots \dots [7b]$$

$$\text{Per cent A pptd.} = 100 - \frac{2(100 - \%S)^4}{\left(100 - \frac{\%S}{2}\right) \left[\left(100 - \frac{\%S}{2}\right)^2 + (100 - \%S)^2\right]} \dots [11b]$$

The percentages of A precipitated by increasing percentages of S III, calculated according to these equations, are given in Table VII. These data are shown graphically in Fig. 3, in which Curve A is calculated according to [7 b] and Curve B according to [6 b].

In Table VIII the data on Serum 607 in Table I are calculated in terms of percentage of the total precipitated at the equivalence point, and the percentage of S III used and antibody N precipitated are plotted on Fig. 3. It will be seen that the circles representing the values found at 0° lie very close to the curve (A) for the reaction in which the N : S ratio varies from R to 3 R (equation [7 b]) while the 37° values are very near those calculated for the R to 2R reaction (equation [6 b], Curve B). Thus the course of the reaction at 0° appears to be determined by a greater complexity of the reactions occurring after the initial A and S combination than is indicated by the data for the reaction at 37° .

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If, instead of using the equivalence point as the basis of the calculation, the ratio at the beginning of the equivalence zone (from the region of excess antibody) be used, the course of the reaction follows the two stage expression [6 a] very closely in all but one of the anti-

TABLE VII
Calculated Percentage of Antibody Precipitated

Total S III added Calculated according to..... Ratio limits.....	Antibody N precipitated		
	Equation 6b	Equation 11b	Equation 7b
	R and 2 R	R and 2 R	R and 3 R
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
10	19.0	19.4	27.5
20	36.0	37.2	50.0
30	51.0	53.4	67.8
40	64.0	67.6	80.9
50	75.0	79.5	90.0
60	84.0	88.7	95.6
70	91.0	95.1	98.5
80	96.0	98.7	99.7
90	99.0	99.88	99.98
100	100.0	100.0	100.0

TABLE VIII
*Antibody N Precipitated by S III from Serum 607
Expressed as Percentage of Quantity Precipitated at Equivalence Point*

S III used	Reaction at 0°			Reaction at 37°		
	S III	Antibody N pptd.	Antibody N pptd.	S III	Antibody N pptd.	Antibody N pptd.
	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
<i>mg.</i>						
0.02	14.5	0.62	42.8	15.8	0.42	31.6
0.04	29.0	1.03	71.0	31.5	0.74	55.6
0.06	43.5	1.25	86.2			
0.075	54.4	1.35	93.1			
0.09	65.2	1.40	96.6	59.1	1.16	87.2
0.10	72.5	1.43	98.6	70.9	1.23	92.5

body solutions studied in sufficient detail, regardless of the temperature at which the reaction is carried out.⁹ The theoretical amounts

⁹ The exception, BX, is one of the solutions studied in the beginning of the work (1), in which aliquot portions of supernatant were analyzed instead of entire precipitates. It was, moreover, an exceedingly concentrated solution.

of antibody nitrogen precipitated by varying quantities of S III from different antibody solutions according to equation [6 *a*] were calculated with the aid of the experimental values for *R* given in Table VI for the ratio at the beginning of the equivalence zone, *A* being nitrogen precipitated at this point. A comparison is given in Table IX of the calculated and experimental values for nitrogen precipitated.

In a previous paper (3) it was shown that the antibody nitrogen precipitated by S III from Solution B 31 in the region of excess antibody followed the empirical equation, $N = 18.6 S - 60 S^2$. It will be noted that this equation is in the same form as [6 *a*], so that the theoretical significance of the two constants is now clear, for $18.6 = 2R$ and $60 = \frac{R^2}{A}$.

The results of the serial experiments (Table II) also conform to equation [6]. In order to make the comparison with other data, the result from each successive addition of S III was calculated to the 1.0 cc. basis. The ratio of total antibody nitrogen precipitated to total S III used was taken as *R* and the total antibody nitrogen per 1.0 cc. of antibody solution as *A* in the equation [6 *a*]. Curves A and B, Fig. 1, were calculated with the aid of these values. The circles along the curves represent the actual experimental data.

In the region of excess S III, by interchange of *S* and *A* (page 581), equation [6 *a*] becomes:

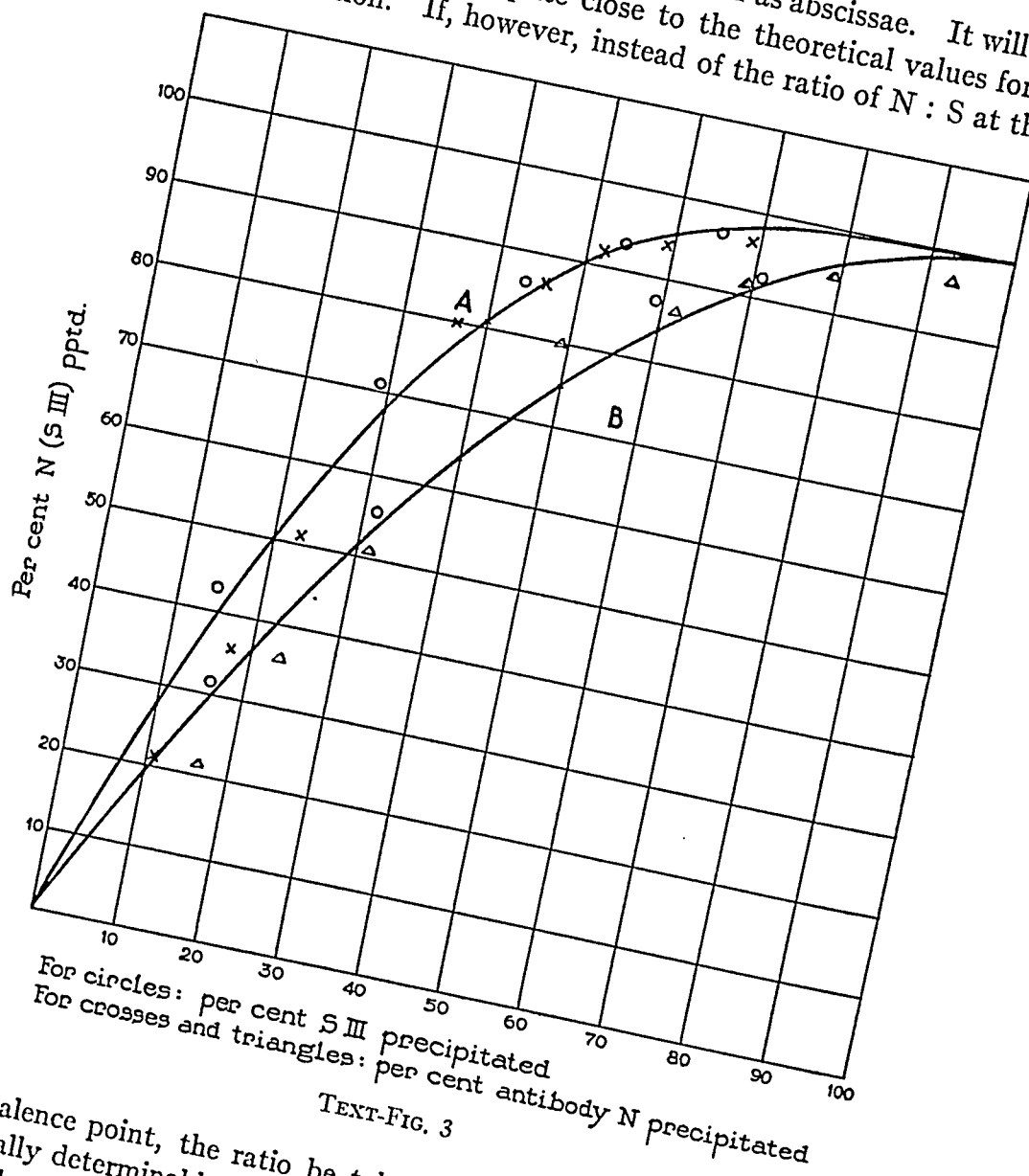
$$\text{mg. S III precipitated} = 2 R' A - \frac{(R')^2 A^2}{\text{Total S}} \dots\dots\dots [12],$$

in which $R' = \frac{S}{A}$ at the equivalence point.

Thus, as in equation [6], the amount of S precipitated depends only on the relative amounts of *S* and *A* present. Expressions similar to [12] may also be found corresponding to [6 *b*], [7 *a*], and [7 *b*] by interchanging *A* and *S*. In making calculations according to these equations 100 per cent *A* was taken as $\frac{\text{Total S III present}}{R'}$, or the amount which would combine with the S III present to form the equivalence point compound. The points obtained from the data in Table III are plotted as crosses in Fig. 3, using the per cent of S III pre-

QUANTITATIVE PRECIPITIN STUDY

precipitated as ordinates and the per cent of A as abscissae. It will be seen that the values fall quite close to the theoretical values for a three step reaction. If, however, instead of the ratio of N : S at the



TEXT-FIG. 3

equivalence point, the ratio be taken as 7.5, at which point an analytically determinable amount of S III first appears in the supernatant, the reaction follows the two stage mechanism, according to equation [6 b]. This procedure is analogous to that used in the region of

excess antibody. The points derived in this way are plotted as triangles in Fig. 3. The three lowest points are partially in the inhibition zone and could scarcely be expected to conform closely to the curves. Similar considerations apply to antibody Solution B 62 at 0°, for which the data are also given in Table III.

TABLE IX

Comparison of Experimental Data with Values Calculated According to:

$$N \text{ Precipitated} = 2RS - \frac{R^2S^2}{A}$$

Antibody No.....	BV _A		B 36		B 61		B 62		B 62		Serum 607		Serum 607	
Temperature, °C...	37, 0		37, 0		37, 37		0, 0		37, 37		0, 0		37, 37	
R.....	13.6		12.4		11.4		(17)		12		(15)		(11)	
A.....	4.08		1.86		1.71		(1.23)		1.20		(1.42)		(1.31)	
S III used	N pptd.		N pptd.		N pptd.		N pptd.		N pptd.		N pptd.		N pptd.	
	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0.01							0.36	0.32						
0.02			0.50	0.46	0.45	0.43	0.57	0.59	0.44	0.43	0.62	0.54	0.42	0.40
0.03							0.78	0.81						
0.04					0.79	0.79					1.03	0.95	0.74	0.73
0.05	1.22	1.25	1.03	1.03	0.97	0.95	1.07	1.11						
0.06					1.05	1.09			0.96	1.01	1.25	1.23		
0.075			1.41	1.40							1.35	1.36	1.16	1.13
0.08					1.29	1.34								
0.09											1.40	1.42	1.23	1.23
0.10	2.24	2.27	1.66	1.65	1.54	1.52								
0.12					1.68	1.64								
0.20	3.62	3.62												
0.25	3.87	3.96												

R and A values in parentheses deduced from nearest actual determination.

A relationship useful in its application to unknown sera may be derived from expression [6 a]. If both sides of the equation be divided by S, the resulting equation, $\frac{N}{S} = 2R - \frac{R^2}{A} S$, is that of a straight line. Thus, if the values of the ratio found in the region of excess antibody are plotted as ordinates against the amounts of S III added as abscissae, a straight line is obtained. The intercept on the

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y axis gives the value of $2R$, while the slope is $\frac{R^2}{A}$, from which A , the amount of nitrogen precipitated at the beginning of the equivalence zone, may be calculated. Line F, Fig. 2 (page 576), illustrates this

TABLE X
Means of Linear Relation $\frac{N}{S} = 2R - \frac{R^2}{A} S$

Antibody No.....		BVA		B 36		B 61		B 62		Serum 607	Serum 607
Temperature, °C....		37, 0		37, 0		37, 37		0, 0		0, 0	37, 37
S III added at 2 points used, mg....		0.10, 0.20*		0.05, 0.10*		0.05, 0.10*		0.03, 0.05*		0.04, 0.075*	0.04, 0.075*
Values given by line drawn through the 2 points		$2R$		$2R$		$2R$		$2R$		$2R$	
		$\frac{R^2}{A}$		$\frac{R^2}{A}$		$\frac{R^2}{A}$		$\frac{R^2}{A}$		$\frac{R^2}{A}$	
		26.8		24.6		23.4		33		34.8	
		43.5		80		80		233		224	
		N pptd.		N pptd.		N pptd.		N pptd.		N pptd.	
		Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
A.....		4.08	4.13	1.86	1.89	1.71	1.71	1.17	1.35	1.40	1.40
Amt. S III mg.											
0.01											
0.02											
0.03											
0.04											
0.05											
0.06											
0.075											
0.08											
0.09											
0.10											
0.12											
0.20											
0.25											
		1.22	1.23	0.50	0.46	0.45	0.44	0.36	0.31	0.62	0.42
				1.03*	0.79	0.97*	0.81	0.57	0.57	1.03*	0.74*
				1.41	1.08	1.12	1.07*	1.25	1.28	1.16*	1.28
				1.66*	1.29	1.36		1.35*	1.32	1.23	
					1.54*	1.68	1.66	1.40			
		3.62*	3.98								
		3.87									

* These points are also marked with an asterisk in the N pptd. columns below.

procedure in the case of antibody Solution B 61, the circles being the experimentally found ratios in Column 3, Table I. The calculated ratios at the beginning of the equivalence zone given in Column

3 of Table VI were obtained in this way and are probably more accurate than the observed ratios because the experimental errors in the determination of the individual points are averaged in this method.

This linear relationship makes it possible to characterize an unknown Type III antipneumococcus serum or antibody solution in the region of excess antibody by two analyses, in duplicate. If the ratio of antibody N to S III precipitated be determined for two different amounts of S III in the region of excess antibody and a straight line be drawn through the two points so obtained, the intercept on the y axis = $2R$ and the slope = $\frac{R^2}{A}$. With the R and A values at the beginning of the equivalence zone calculated in this way the amount of antibody nitrogen precipitated by any quantity of S III less than $\frac{A}{R}$ may be calculated with a fair degree of accuracy. In choosing the amounts of S III to be used in the determination of these points it is best to precipitate more than 50 per cent of the antibody, since above this level experimental errors in the determination of nitrogen have a smaller effect on the $\frac{N}{S}$ ratio. The application of this procedure to several antibody solutions is illustrated in Table X. It will be seen that there is in general good agreement between the observed and calculated points, but it is better, of course, to have three points with which to determine the position of the line.

In Fig. 2 and in making calculations in the region of excess antibody it is assumed that all of the antibody present is precipitated at the beginning of the equivalence zone. The data in Reference 1 and in Tables I and III show that this is actually not the case, and that the amount of antibody precipitated usually increases as S III is increased in the equivalence zone, often reaching its maximum only when S III is present in appreciable excess. In different sera the amount of additional antibody nitrogen precipitated in this way varies from a few hundredths to one- or two-tenths of a milligram. This behavior appears due to the varying amounts of the relatively easily dissociable antibody occurring in different sera, and renders necessary for the complete description of the behavior of a serum in the precipitin reaction a separate determination of the maximum amount of specifically precipitable nitrogen (3, 4, 7).

QUANTITATIVE PRECIPITIN STUDY

In the region of excess S III the behavior of a serum as far as the beginning of the inhibition zone may be characterized by the determination of the A and S III precipitated at two points, since in this region the linear relation $\frac{S_{pptd.}}{A} = 2R' - \frac{(R')^2 A}{\text{Total } S}$ applies if R' be taken as the $\frac{S}{A}$ ratio at the end of the equivalence zone at which S III appears in excess and A be taken as the amount of antibody precipitated.

In the inhibition zone, in which large amounts of S III are present and the amount of precipitate has begun to diminish, this equation is no longer applicable and it is necessary to determine the dissociation constant of the soluble compound AS_z according to the method indicated in Table V, Reference 1. The determination of two properly spaced points should be sufficient to establish the dissociation constant and permit the calculation of other points in this range.

SUMMARY AND CONCLUSION

The precipitin reaction between the specific polysaccharide of Type III pneumococcus and homologous antibody formed in the horse can be accounted for quantitatively by assuming the chemical combination of the components in a bimolecular reaction, followed by a series of competing bimolecular reactions which depend upon the relative proportions of the components. These reactions would lead to the formation of larger and larger aggregates until precipitation ultimately occurred. The mathematical formulation of this theory on the basis of the mass law is described. The derived expressions are shown to be in accord with the experimental findings and the constants used in these expressions are shown to have definite significance. In spite of the wide variation in the properties of individual sera these expressions permit the complete description of the behavior of an unknown serum with S III without an unduly burdensome number of analyses.

The quantitative theory presented has been found applicable to other instances of the precipitin reaction, as will be shown in subsequent papers.

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THE RELATION OF LEUKOSIS TO SARCOMA OF CHICKENS*

I. SARCOMA AND ERYTHROLEUKOSIS (STRAIN 13)

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PLATES 24 TO 28

(Received for publication, January 16, 1935)

All known causative agents of leukosis and sarcoma of chickens stimulate either a single type of cell or several closely related types of cells to unrestricted multiplication (1, 2). The association of sarcoma with leukosis was noted by us in 1930 among chickens inoculated with our leukosis Strain 1 and also as a spontaneous disease (3); but the etiological relationship of these two diseases was not determined. There was no evidence that the agent of leukosis Strain 1 is capable of producing both sarcoma and leukosis. McIntosh (4) has shown recently that treatment with tar is followed by the development of neoplasms that are transmissible by filterable viruses. This finding suggests that viruses that produce neoplasms are widespread among chickens.

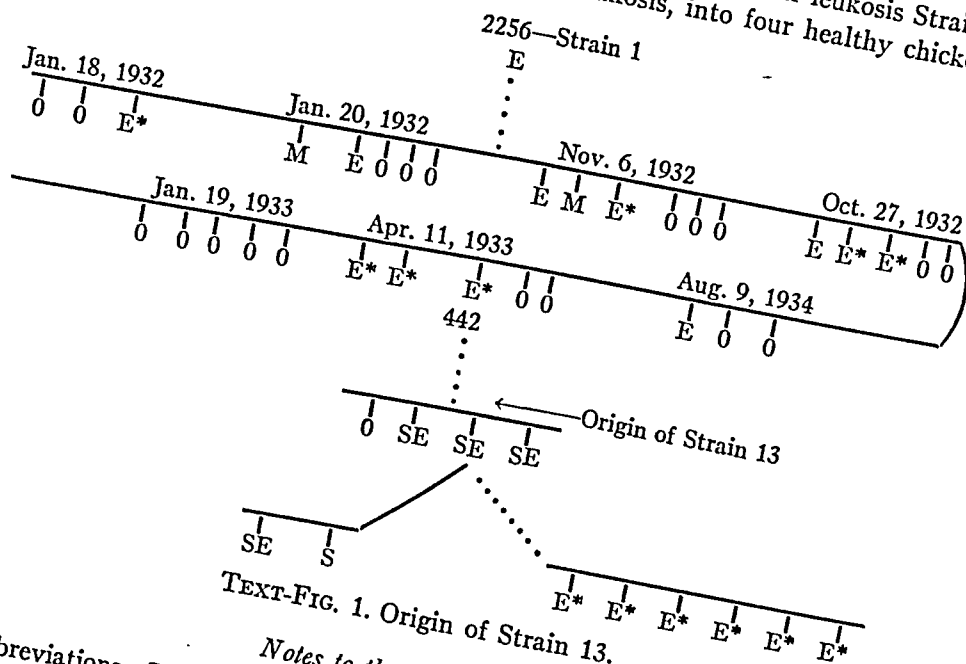
Oberling and Guérin (5) described an agent that in their opinion is capable of producing both leukosis and sarcoma, and probably also carcinoma. They suggest that the common agent of leukosis may "mutate" into an agent with affinity for both mesodermal and ectodermal tissues. Their experiments have been repeated with the same results by Troisier (6) and partially confirmed by Rothe Meyer and Engelbreth-Holm (7), the latter workers having described a strain that produces sarcoma and leukosis but not carcinoma. There are no reports on the incidence of neoplasms among the uninjected control chickens by any of these workers. The experiments described here were undertaken to determine whether a transmissible disease of

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LEUKOSIS, AND SARCOMA OF CHICKENS. I

fowls, known as Strain 13 is caused by one or several viruses. This strain is characterized by the formation of endothelial neoplasms in the blood-forming organs often associated with erythroblastic proliferation.

One of us (May, 1933) made intravenous passages with our leukosis Strain 1, from a chicken (No. 442) that had erythroleukosis, into four healthy chickens.



TEXT-FIG. 1. Origin of Strain 13.

Notes to the Text-Figures

Abbreviations: S = sarcoma, E = erythroleukosis, M = myeloid leukemia,
0 = inoculation unsuccessful.

Route of injections: | = intramuscular, ∴ = intravenous, | = intramus-
cular and intravenous.

*Microscopic examination was omitted.

In addition to erythroleukosis, two of the inoculated chickens developed sarcoma at the site of intravenous injection. One developed erythroleukosis in association with sarcomatosis of the blood-forming organs, and the fourth was apparently unaffected (Text-fig. 1). Since that time more than thirty-five passages have been made with either blood or tumor tissue and the inoculated birds developed sarcoma or sarcoma in combination with erythroleukosis. The diseases transmissible with material deriving from Fowl 442 will be named Strain 13.

Fowl 442, from which Strain 13 originated, was injected intravenously with dried blood of No. 2256 on Apr. 4, 1933. Jan. 18, 1932, three chickens were

injected with fresh blood of No. 2256, and one of them developed erythroleukosis. From Jan. 20, 1932, to Aug. 9, 1934, twenty-nine chickens were injected with dried blood of No. 2256, of which twelve developed erythroleukosis or myeloid leukemia as indicated by the examination of the blood and by the gross findings at necropsy. The blood-forming organs from six of these chickens were studied microscopically and showed leukosis unassociated with sarcoma (Text-fig. 1). No. 442 was not examined microscopically. Although the blood smear and gross postmortem findings of this chicken were characteristic of erythroleukosis, its blood produced sarcoma about the injected wing vein in two of the three chickens that were inoculated (Text-fig. 1). These data indicate that Strain 13 arose in No. 442. The agent of Strain 1 has either assumed the ability to produce sarcoma or a sarcoma virus present in No. 442 has contaminated it.

We have assumed that in Strain 13 we were dealing with a mixture of two agents and have attempted to separate them. The problem proved to be intricate. The agent of this sarcoma strain had an affinity for connective tissue cells as well as for endothelial cells of the blood-forming organs (bone marrow, spleen, and liver) and affected most chickens, whether injected intramuscularly or intravenously. In most instances of erythroleukosis that were studied microscopically, the small sample of marrow examined showed diffuse sarcomatosis or endothelial proliferation with a profound derangement of the vascular bed of the marrow; but sarcoma of the injected breast muscle was often found unassociated with erythroleukosis. It seemed doubtful whether the erythropoietic changes produced by Strain 13 were identical with the classical erythroleukosis described by Ellermann, for they may have been secondary to the endothelial lesions caused by this virus. In most instances they could be readily distinguished on gross postmortem examination from primary erythroleukosis. Erythroleukosis of Ellermann is characterized by progressive, seemingly autonomous proliferation of erythroblasts that invade the blood and accumulate in the pulp of the spleen and in the capillaries of many organs; e.g., liver, lung. Retarded or arrested maturation and leukostasis distinguish erythroleukosis from secondary erythroblastic hyperplasia, the latter being associated with normal maturation of erythroblasts and with no leukostasis. Most instances of erythroleukosis produced by Strain 13 are modified by coexistent diffuse endothelial neoplasms, but pure instances of erythroleukosis of the bone marrow occur among the passages of this strain and histological

studies strongly support the view that the erythroleukoses produced by Strain 1 and Strain 13 are essentially the same disease.

Anatomical Characteristics

Tumors produced with intramuscular inoculations of this strain (Fig. 1) are firm and exude large amounts of mucinous material. They often contain cysts filled with such material (Fig. 7), and hemorrhages. They grow rapidly, and 2 to 3 weeks after injection into young chickens may occupy most of the breast muscle. Softening and hemorrhages into the surrounding tissues frequently occur. Microscopically, the tumors are composed of cells that resemble closely those of the common spindle cell sarcomata (Fig. 8) and occasionally they contain multinuclear giant cells (Fig. 9). Yet it is doubtful whether these cells are of connective tissue origin, because the malignant cells of the tumor caused by the virus of Begg and Murray in the breast muscle are similar to those produced by Strain 13 but have been traced to endothelium by these observers.

On gross examination internal sarcomatosis was usually suggested by hematmata, with no gross tumors, or occasionally gray tumor nodules or diffuse infiltrations. The liver, spleen (Fig. 3), and bone marrow (Fig. 4), and the gonads were the most frequent sites of neoplasms whose endothelial character was indicated by continuity with endothelium, and presence of blood in channels formed by tumor cells. Microscopically, proliferations of endothelial cells associated with derangement of the vascular system were usually but not always found at the site of the hematmata. Diffuse sarcomatosis of the spleen with replacement of the lymphoid tissue and dilatation of the sinuses is shown in Fig. 11. Neoplastic proliferation of endothelial cells in the lumen of a blood vessel of the spleen is shown in Fig. 12.

Erythroblastic proliferation caused by Strain 13 is, in most instances, readily distinguishable from that produced by the common leukosis strains (e. g. our Strain 1) by gross examination of either the bone marrow or the spleen. In the usual type of erythroleukosis of Ellermann, the bone marrow is firm, red, and can be removed as a solid cylindrical mass, but in Sarcoma 13 the marrow is usually soft, "watery," and hemorrhagic (Fig. 4). The spleen in the more common erythroleukosis (Fig. 2) is greatly enlarged and is uniformly red, whereas in Strain 13 it is only slightly or moderately enlarged, and is gray because of replacement with tumor tissue; or it contains one or several hematmata (Fig. 3).

Intravenous inoculation in very young chickens is frequently followed by conspicuous weakness of the legs. This is apparently due to extensive hemorrhage in the bone marrow (Fig. 4) that can often be seen through the intact cortex of femur and tibia, and occasionally separates the proximal epiphysis from the diaphysis. A tumor that measured about 2 x 1 x 0.5 cm., and had invaded the cortex of the tibia, was found in a young chicken (Fig. 4).

Sections of the bone marrow from 52 chickens that were successfully inoculated with Strain 13 were examined.

(a) 17 showed neither erythroleukosis nor sarcoma of the bone marrow; (b) 21 showed both; (c) 6 showed evidence of sarcomatosis of the bone marrow; (d) 7 showed evidence of erythroblastic proliferation resembling erythroleukosis of Ellermann, and sarcoma in some organ other than bone marrow; (e) 1 showed erythroleukosis unassociated with sarcomatosis. Thus only one (No. 73) of the chickens studied microscopically had pure erythroleukosis indistinguishable from that produced by Strain 1. In addition, however, seven chickens (group *d*) showed erythroblastic proliferation of the bone marrow unassociated with maturation of erythroblasts and sarcoma of the muscles, spleen, or some other organ. In four of these birds erythroleukosis was advanced, in three it was slight. Several birds had hematmata in organs other than the bone marrow but the erythroblastic hyperplasia of the marrow, unlike that which follows loss of blood, was unassociated with maturation and was indistinguishable from that which characterizes incipient erythroleukosis (Fig. 2 (8)). These findings strangely support the assumption that the virus of Strain 13 may stimulate independently erythroblasts as well as endothelial cells. It is possible that parts of the unexamined marrow of some of the eight chickens included in groups *d* and *e* had sarcomatous changes, but it does not seem probable that this is true for all members of these groups. Moreover, the occurrence of hemorrhage or sarcoma in one part of the bone marrow does not explain proliferation of erythroblasts with arrested maturation in other parts of the marrow.

Sarcomata were found in the ovary, testis (Fig. 10), and less frequently in the lung, kidney, skin (Figs. 15-17), and heart. Hemorrhage from spleen or liver was often the immediate cause of death.

The histogenesis of the changes produced by this strain needs further investigation. The tumors produced in the breast muscle resemble those of connective tissue origin (Figs. 7-9), while most neoplasms found in the skin and the internal organs appear to arise *in situ* from endothelium. Examples of endothelial neoplasms are shown in Fig. 5 (bone marrow), Figs. 15-17 (skin), Figs. 13 and 14 (ovary). Endothelial hyperplasia, such as shown in Fig. 18, was often found in the liver of chickens injected with Strain 13. Side by side with distinctly endothelial neoplasms there were solid masses of sarcoma cells whose origin was not determined. The changes in the spleen, characteristic for Strain 13 and illustrated in Figs. 11 and 12, have the morphological characteristics of a neoplasm that has arisen through malignant transformation of endothelial cells of the spleen. Similar alterations were found in the bone marrow, ovary (Figs. 13, 14), and testis.

The formation of blood cells from endothelial cells was not seen in any of these preparations. The channels lined with neoplastic endothelium either contained normal blood cells (Figs. 5, 18) or, in the presence of coexistent erythroleukosis, they contained basophile erythroblasts (Figs. 10, 13, 14).

Transmission Experiments

A survey of the passages made with Strain 13 is shown in Text-figure 2. In this figure only those chickens are recorded (fifth to the eighteen subpassages) from which further subpassages were made and details of significant experiments will be given in the text.

Text-fig. 2 resembles the similar figure of Rothe Meyer and Engelbreth-Holm which shows the passages of their strain that produces both sarcoma and leukosis. These authors found that intravenous transmission produces erythroleukosis almost exclusively while intramuscular transmission produces sarcoma occasionally associated with erythroleukosis. The results of our first five passages appeared to confirm fully the findings of Rothe Meyer and Engelbreth-Holm, until it was discovered that the chicken inoculated intravenously and believed to have erythroleukosis only, had also diffuse endothelial sarcomatosis of several organs. Diffuse endothelial neoplasm of the blood-forming organs has never before been observed in the chicken, so far as we are aware, and distinguishes the avian sarcomata thus far described from our Sarcoma 13.

Intramuscular Inoculations.—Oberling and Guérin believe that the type of disease produced by the common agent of leukosis and sarcoma depends mainly on the route of introduction, and that we have failed to note the ability of our leukosis Strain 1 to produce sarcoma because our inoculations were intravenous. This, however, is not the case, for our leukosis Strains 1, 2, and 5 do not produce spindle celled or fibroblastic tumors resembling the Rous sarcomata after intramuscular inoculations. The intravenous route of transmission of Strain 1 was chosen by us after preliminary experiments had made evident that leukosis Strain 1 is best transmitted by intravenous inoculations. The causative agent of leukosis Strain 1 after intramuscular inoculations produces systemic disease (erythroleukosis or, less often, myeloblastic leukemia) with no tumors at the site of injection; but most adult chickens inoculated in this manner remain healthy. Sarcomata such as those studied by Rous, on the contrary, are often readily transmitted by intramuscular injections, and it is possible that if this route of introduction is practiced with a mixed virus of leukosis and a sarcoma, it may occasionally lead to the isolation of the sarcoma virus. This, however, was not observed. Successive intramuscular inocula-

Passage	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
	3914 SE a/b	3970 S(E?) c	3971 SE b	3972 S f, g	66 SE c	70 E* c	73 E i	76 E* j	79 E* k	3913 S d	4611 S d	4639 S g	4703 SE i
	3977 S a	2960 S c	4040 S c	3518 S a	3518 S a	4366 S a	4170 SE c, d	4258 SE d, c	4526 S(M?) b	4525 S a	230 S a	4650 SE a	4701 S(E?) a
	3110 S a, b	3116 S d	4139 S b	4304 S b	4138 S f	4304 S b	190 S c	2277 S l	300 S c	319 S k	4080 S j	305 SE k	293 SE g, h
	4370 S c	4303 S d	4169 S c	4170 SE c, d	4169 S c	4366 S a	4501 SE a	4525 S a	4590 S(E?) b	183 SE b	185 S b	241 S b	263 S a
	4164 SE g	4170 SE c, d	4169 S c	4170 SE c, d	4169 S c	4366 S a	4501 SE a	4525 S a	4590 S(E?) b	183 SE b	185 S b	241 S b	263 S a
	3108 S j	4474 SE b	190 S c	2277 S l	300 S c	319 S k	4080 S j	305 SE k	293 SE g, h	4704 SE j	4703 SE i	4703 SE i	4703 SE i
	322 SE f, g	4474 SE b	190 S c	2277 S l	300 S c	319 S k	4080 S j	305 SE k	293 SE g, h	4704 SE j	4703 SE i	4703 SE i	4703 SE i

TEXT-FIG. 2. Passages of Strain 13.

tions, examples of which are shown in Text-figs. 3 and 4, produced either seemingly pure sarcoma or sarcoma in combination with erythro-leukosis; but among the passages made from chickens with apparently pure sarcoma, there occurred again sarcoma and leukosis. Most chickens with metastatic sarcoma of the bone marrow showed the blood picture of either anemia or erythroleukosis.

Sarcoma 13 was usually associated with extensive hemorrhages, and the microscopic differentiation of erythroleukosis from secondary anemia was difficult. It is possible that the small sample of marrow taken for microscopic examination was normal, while foci of sarcoma or leukosis were present in parts of the unexamined marrow. It is also possible that a chicken with no evidence of erythroleukosis after inoculation still carried the introduced virus. However, with few exceptions, when the blood smear indicated anemia or erythroleukosis, microscopic examination of the marrow showed diffuse sarcomatosis with or without erythroblastic hyperplasia. The reverse was also true; when the circulating blood appeared normal, the marrow showed only slight or no evidence of sarcomatosis.

Successive intravenous subpassages (see Text-fig. 2), with blood of chickens that showed anemia or erythroleukosis, have likewise failed to isolate the agent of erythroleukosis; but all chickens successfully inoculated by the intravenous route and examined microscopically, showed, with two exceptions, sarcomatosis of the blood-forming organs.

Chickens inoculated intramuscularly and intravenously with minute amounts of blood (0.00001 cc.) from a chicken that had both sarcoma and erythroleukosis, developed seemingly pure erythroleukosis. A similar subpassage with minute amounts of blood (0.00001 cc.) from one of these chickens, yielded again seemingly pure erythroleukosis. Inoculation with a large amount of blood (1 cc.) from the same chicken produced, however, sarcoma in the injected breast muscle. These puzzling results were fully explained by the microscopic examination which showed that the chicken injected with 0.00001 cc. of blood and believed to have pure erythroleukosis, also had an extensive diffuse sarcomatosis of the blood-forming organs. These experiments are more fully described below. Successive intravenous subpassages failed to yield a virus that would produce erythroleukosis only. It

cannot be expected to do so because intravenous inoculation produces diffuse sarcomatosis of the internal organs as regularly as intramuscular injection produces sarcoma of the injected muscle.

Attempts to Separate the Hypothetical Sarcoma Agent from the Hypothetical Leukosis Agent by Injecting Diminishing Amounts of Blood or Tumor Tissue

If Strain 13 is composed of a mixture of two agents and if there is a difference in the effective concentration of these agents in a given inoculum, then by injecting diminishing amounts of it one should reach a dose that produces only sarcoma or leukosis. Since the concentration of the transmitting agent of leukosis in the blood is variable and may be as high as one millionth of a cubic centimeter (9) and each dose has to be tested on several chickens, the experiments reported here cannot be regarded as complete.

Intramuscular Titration.—The blood of a chicken that had sarcoma and leukosis was inoculated in diminishing amounts into the muscles of the left wing and left breast of six chickens, with the following results (Passage X *z*).

Chicken No.	Amount injected	Period of observation	Lesion
	cc.	days	
275	0.1	D 16	Sarcoma and erythroleukosis
274	0.1	D 30	Sarcoma
273	0.001	D 26	Sarcoma and erythroleukosis
272	0.001	D 24	Sarcoma and erythroleukosis
271	0.00001	D 21	Erythroleukosis
270	0.00001	D 30	Erythroleukosis

Abbreviations Used in the Tabulations

D = died, K = killed. The figures that follow the letters D or K show the period of observation, in days, after inoculation; e. g. "D 28" denotes that the chicken died 28 days after inoculation.

Route of injection: i.m. = intramuscular, i.v. = intravenous.

Most of the chickens used were young Barred Rocks, or White Leghorns; their age varied from a few to about 100 days.

Intramuscular injection of 0.001 cc. of blood produced both erythroleukosis and sarcoma, but 0.00001 cc. produced erythroleukosis only, with no tumor at the site of injection. No tissues were taken for microscopic examination from the

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Sarcoma 13 was usually associated with extensive hemorrhages, and the microscopic differentiation of erythroleukosis from secondary anemia was difficult. It is possible that the small sample of marrow taken for microscopic examination was normal, while foci of sarcoma or leukosis were present in parts of the unexamined marrow. It is also possible that a chicken with no evidence of erythroleukosis after inoculation still carried the introduced virus. However, with few exceptions, when the blood smear indicated anemia or erythroleukosis, microscopic examination of the marrow showed diffuse sarcomatosis with or with no erythroblastic hyperplasia. The reverse was also true; when the circulating blood appeared normal, the marrow showed only slight or no evidence of sarcomatosis.

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Intramuscular Titration.—The blood of a chicken that had sarcoma and leukosis was inoculated in diminishing amounts into the muscles of the left wing and left breast of six chickens, with the following results (Passage X i).

Chicken No.	Amount injected	Period of observation	Lesion
	cc.	days	
275	0.1	D 16	Sarcoma and erythroleukosis
274	0.1	D 30	Sarcoma
273	0.001	D 26	Sarcoma and erythroleukosis
272	0.001	D 24	Sarcoma and erythroleukosis
271	0.00001	D 21	Erythroleukosis
270	0.00001	D 30	Erythroleukosis

Abbreviations Used in the Tabulations

D = died, K = killed. The figures that follow the letters D or K show the period of observation, in days, after inoculation; c. g. "D 28" denotes that the chicken died 28 days after inoculation.

Route of injection: i.m. = intramuscular, i.v. = intravenous.

Most of the chickens used were young Barred Rocks, or White Leghorns; their age varied from a few to about 100 days.

Intramuscular injection of 0.001 cc. of blood produced both erythroleukosis and sarcoma, but 0.00001 cc. produced erythroleukosis only, with no tumor at the site of injection. No tissues were taken for microscopic examination from the

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chickens with seemingly pure erythroleukosis. Subpassage was made from one of these chickens (No. 271) by injecting 0.00001 cc. of its blood into the wing vein and pectoral muscle of three chickens (Passage XI *h*). These chickens also died of erythroleukosis in from 36 to 50 days after the inoculation with no grossly detectable tumors in the injected breast muscle. The microscopic examination of one of these chickens (No. 293) showed extensive diffuse sarcomatosis of the blood-forming organs. At the time these experiments were made the significance of internal sarcomatosis was not appreciated and, with the exception stated, the diagnosis was based solely on blood smears and on gross postmortem examination. It now appears highly probable that all chickens of this series had sarcomatosis of the bone marrow because of the multiple hematomata noted in the gross in these organs. When large amounts of blood (1 cc.) from No. 293 were injected intramuscularly into each of two chickens, sarcoma developed in both at the site of injection (Passage XII *g*). Microscopic study revealed that No. 293 had internal sarcomatosis as well as erythroleukosis.

These experiments indicate that the blood of chickens with internal sarcomatosis transmits the disease in amounts as small as 0.00001cc. The results now to be described suggest that the intravenous route of injection may be superior to the intramuscular route for the demonstration of small amounts of virus.

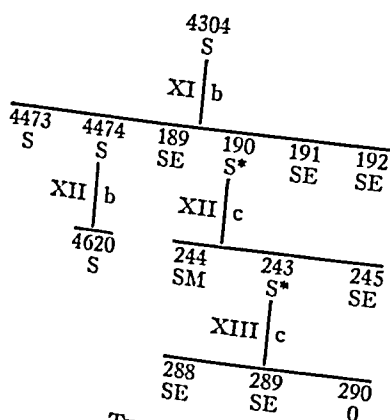
In an earlier experiment (Passage IX *d*) diminishing amounts of dried blood from a chicken (No. 3116) that had sarcoma and showed no gross or microscopic evidence of leukosis, were injected intramuscularly and intravenously into fifteen chickens with the following results.

Amount injected cc.	Route of injection	Period of observation. Lesion
0.1	i.m.	D 39. Sarcoma and erythroleukosis. K 124. Sarcoma*
0.1	i.v.	D 45. Sarcoma and erythroleukosis
0.01	i.m.	D 84. Sarcoma.* K 84. Negative
0.01	i.v.	D 26. Erythroleukosis.* D 30. Erythroleukosis*
0.002	i.m.	K 84. Negative. K 84. Negative
0.002	i.v.	D 51. Erythroleukosis.* K 90. Negative
0.0002	i.m.	D 56. Negative. K 84. Negative
0.0002	i.v.	K 90. Negative. K 90. Negative

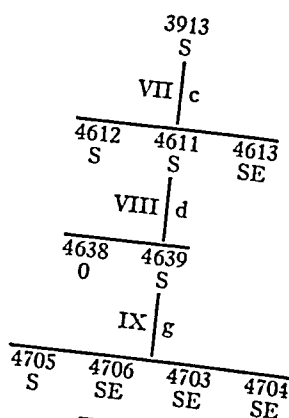
Negative means that there was no evidence of leukosis and sarcoma.
* These chickens have not been examined microscopically.

The results of this experiment were essentially the same as those already described. Small amounts (0.01 and 0.002 cc.) of dried blood

were more active after intravenous than after intramuscular injection. Small amounts of blood appeared to produce erythroleukosis only; larger amounts of blood produced both leukosis and sarcoma. We now believe that all of these chickens with "pure" erythroleukosis had, in addition, an internal sarcomatosis. In the previous experiment, fresh blood of leukotic chickens was used for inoculations; in the present experiment, dried blood was used from a chicken that during life showed no evidence of leukosis, and on postmortem examination appeared to be free of leukosis.



TEXT-FIG. 3



TEXT-FIG. 4

TEXT-FIGS. 3 and 4. Results of successive intramuscular inoculations.

A similar experiment (Passage VIII *f, g*), made with sarcoma obtained from a chicken (No. 3972) that showed no gross or microscopic evidence of leukosis, was less successful. Three chickens injected intravenously with 0.5 cc. of blood remained healthy; but one of two chickens that were injected intravenously with 0.5 cc. of a tumor extract died of erythroleukosis. Also, one of two chickens injected intravenously with 0.01 cc. of the same tumor extract died of erythroleukosis.

These experiments show that small amounts of blood or tumor tissue inoculated intravenously produce erythroleukosis, and larger amounts inoculated intramuscularly produce sarcoma at the site of injection, even though the chicken whose tissues were used for inoculation had sarcoma unassociated with erythroleukosis. These results strongly support the view that the same agent that produced sarcoma is responsible for the development of erythroblastic proliferation.

Inoculation of Chickens Immune to Leukosis

Chickens that are resistant to one inoculation with tissues containing leukotic cells are, with rare exceptions, resistant to reinjections with similar material (10) and can therefore be regarded as immune to leukosis. Most chickens immune to leukosis Strain 1 were resistant to inoculations with leukosis Strain 2 (10).

In the next experiments, chickens that were resistant to repeated injections with leukotic tissues were injected with Strain 13 and developed sarcoma unassociated with leukosis. This procedure appeared to be promising in the separation of the assumed agents of sarcoma and of leukosis; but in the subpassages made from such chickens leukosis invariably made its appearance.

Experiment 1.—(Passage VIII b.) Five chickens that resisted repeated inoculations with tissues of leukotic chickens were injected intramuscularly with tumor tissue of Strain 13. All five chickens developed sarcoma unassociated with leukosis. Sarcoma also developed in all of the four injected control chickens, and in one chicken it was associated with erythroleukosis combined with myeloid leukemia. Three control chickens that had sarcoma only, died or were killed within 25 days after injection, whereas the fourth, that also had leukosis, lived for 45 days. It is probable that the first three chickens would have developed blood changes if the rapidly growing tumors had not caused their death soon after the injection. The leukosis-immune chickens were killed 20, 48, 56, 239, and 278 days, respectively, after injection.

The following is a brief history of these immune chickens.

No. 2960 was inoculated June 27, 1932, with blood, leukosis Strain 1; Dec. 29, with plasma, leukosis Strain 1; June 26, 1933, with blood, leukosis Strain 2; Oct. 5, with tumor tissue, sarcoma Strain 13. It was killed Dec. 25 and had two tumor nodules, each measuring about 3 cm. in longest diameter at the sites of injection. There was no evidence of metastases or leukosis.

No. 3116 was inoculated Dec. 3, 1932, with blood, leukosis Strain 2; June 8, with blood, leukosis Strain 2; Oct. 5, with tumor tissue, sarcoma Strain 13. It was killed Nov. 13, when the tumor in the injected breast muscle measured 7 x 3 x 2 cm. There was a somewhat smaller tumor in the injected leg muscles. There was no evidence of metastases or leukosis.

No. 3410 was inoculated Jan. 24, 1933, with dried blood, leukosis Strain 2; June 26, with fresh blood, leukosis Strain 2; Oct. 5, with tumor tissue, sarcoma Strain 13. It was killed Dec. 1, and the postmortem findings were similar to those of No. 3116.

No. 3422 was inoculated Jan. 24, 1933, with blood, leukosis Strain 1; June 26, with blood, leukosis Strain 2; Oct. 5, with tumor tissue, sarcoma Strain 13. A

tumor nodule that was about 1.5 cm. across, developed in the injected muscles Nov. 21, but slowly regressed.

No. 3530 was inoculated June 16, 1933, with blood, leukosis Strain 2, and Nov. 5, with tumor tissue, sarcoma Strain 13. The tumors that developed at the sites of injections measured about $2.5 \times 1 \times 1$ cm., Nov. 24, in both breast and leg muscles, but slowly regressed. The regressing tumor was successfully implanted Jan. 19 in three of four chickens injected. No. 3530 was reinjected intramuscularly with sarcoma Strain 13, but remained healthy.

Experiment 2.—Eight immune and two control chickens were injected with tissue of sarcoma Strain 13. The two control and four of the immune chickens developed sarcoma; the controls also had severe anemia, whereas the blood of the immune chickens appeared normal. One of the immune chickens (No. 3528) died with adenocarcinoma of the oviduct and extensive carcinomatosis of the peritoneum. Three chickens were inoculated with tissues of this tumor but all remained healthy. This was the only carcinoma found among the chickens injected with this strain. There appears to be no etiological relationship between carcinoma and Strain 13.

The following is a brief history of the immune chickens that were successfully reinjected with sarcoma Strain 13.

No. 2277 was inoculated Dec. 9, 1931, and Apr. 18, 1932, with blood, leukosis Strain 1; Dec. 3, 1932, with blood, leukosis Strain 2; May 3, 1933, with tumor tissue, leukosis Strain 2; July 26 and Nov. 1, with tumor tissue, sarcoma Strain 12. The first injection with sarcoma Strain 13, on Jan. 8, 1934, was unsuccessful, but the second injection of tumor tissue of this strain, made Feb. 26, was followed by the development of a tumor in the injected leg muscles. Three of the chickens injected in July with this tumor developed sarcoma as well as erythroleukosis.

No. 3108 was inoculated in a similar manner, twice with blood of leukosis, Strain 2; twice with tumor, sarcoma Strain 12; twice with sarcoma Strain 13. Only the second injection with Sarcoma 13 was successful. The first injection with Strain 13 was made with material of low virulence.

No. 3152. The history of this chicken is similar to No. 3108, but in this immune chicken there were extensive metastatic sarcomata in the liver and kidneys. The spleen and bone marrow appeared normal and there was no evidence of leukosis.

No. 3521 received two intravenous inoculations with leukosis Strain 2 followed by intramuscular inoculations with sarcoma Strains 11 and 12, and finally by intravenous and intramuscular inoculations with Strain 13. Sarcomata were found in the liver and kidney but there was no tumor at the site of intramuscular injection and it is doubtful whether the sarcomata were actually caused by Strain 13.

The history of the four chickens immune to leukosis in which subsequent inoculation with sarcoma Strain 13 was unsuccessful was similar to the histories given above.

The experiments show that resistance to leukemia is not associated with resistance to Sarcoma 13. Chickens immune to leukemia, when inoculated with Strain 13, appeared to develop pure sarcoma, but erythroleukemia invariably occurred in the subpassages made with sarcoma tissue from these chickens. It is probable that some of these leukemia-immune chickens were carriers of the leukemia virus, but we expected that some of them would be free of the virus of leukemia as well as immune to it. In none of these chickens, however, was Strain 13 deprived of its ability to produce leukemia.

The experiments can be interpreted in several ways. It can be assumed that the virus of sarcoma Strain 13 is unrelated to the virus of leukemia Strain 1. The chickens immune to leukemia developed pure sarcoma after intramuscular injection with sarcoma Strain 13, and did not develop erythroleukemia because the sarcoma did not metastasize to the bone marrow. Localization of the disease to the site of injection can be partially explained by old age of the immune chickens, and also by the fact that most of them were destroyed soon after the sarcoma made its appearance. It is possible that successful intravenous inoculation of sarcoma Strain 13 into chickens presumably immune to leukemia would produce erythroleukemia.

Since the presence of live cells prevents the inactivation of the associated virus by immune sera (11), it seemed possible that if chickens immune to leukemia are inoculated with cell-free extract of Sarcoma 13, they might destroy the hypothetical leukemia virus but not the sarcoma virus.

Experiment 3.—In Passage X *b*, four chickens were injected intramuscularly with desiccated tumor tissue of Sarcoma 13 after two unsuccessful inoculations with leukemia Strain 1. All remained healthy, whereas four of five young control birds injected in a similar manner developed tumors at the site of inoculation. 78 days later the same four chickens, immune to leukemia Strain 1, were reinjected for the second time with sarcoma tissue exposed to -30°C . during 30 minutes (Passage X *j*), this time with success. Three of the four chickens developed sarcoma; it regressed in two. Tumor tissue from the third chicken was injected intramuscularly into three chickens and produced only sarcoma (Passage XI *g*), but in the later subpassages, made by intravenous and intramuscular inoculation, erythroleukemia again appeared.

Experiment 4.—Each of six immune and four control chickens received intramuscular injections of dried blood and dried spleen and intravenous injection of

dried blood (Strain 13). All remained healthy and four controls developed sarcoma with anemia or erythroleukosis.

Each dose was approximately 10 mg. Of the six immune chickens injected, three were resistant to leukosis Strain 1, sarcoma Strains 11 and 12; two were resistant to leukosis Strain 2, sarcoma Strains 11 and 12; and one was resistant to sarcoma Strains 11 and 12. Incidentally, this experiment shows that the transmitting agent of Strain 13 present in the blood and spleen, is readily preserved by drying.

These experiments suggest that most chickens immune to leukosis are resistant to the cell-free virus of Sarcoma 13 and that those that are susceptible are unable to deprive the virus of Strain 13 of its ability to produce erythroleukosis. The studies of Andrewes (12) indicate that immunity to the viruses of chicken tumors is not strictly specific. Further investigations are needed to determine whether resistance to a cell-free virus develops in birds with advancing age and whether it can be produced by inoculations with a heterologous virus.

Neutralization Experiments

In some animals that recover from sarcoma, or leukosis, or that have the disease in a chronic form, antibodies that neutralize the free oncogenic agent appear in the blood. The antibodies directed against the agents of sarcomata have been thoroughly investigated by Andrewes (12), who found that they possess some specificity in addition to a group effect. Antibodies active against the agents of leukosis, according to the few observations made (10, 13), also exert group and specific effects. It seemed possible that sera of chickens resistant to repeated inoculations with leukotic tissues would neutralize the hypothetical leukosis component of Strain 13.

The difficulties are numerous in conducting such neutralization experiments with leukosis agents on a scale large enough to be conclusive. In similar experiments with sarcoma a single chicken may serve as a test object for several serum-virus mixtures; in leukosis several chickens are required for each mixture. Some normal sera inhibit the action of the agent (12). The concentration of neutralizing substances in the serum is apparently small, and the determination of the optimal amount of serum and virus required to demonstrate

specific neutralization would require extensive preliminary experiments.

In one experiment (Passage 4304) none of six chickens developed tumors following injection with tumor filtrate, incubated at 37°C. during $\frac{1}{2}$ hour with varying amounts of a pooled serum of six leukosis-immune chickens.

In another experiment (Passage X j) five chickens were injected intramuscularly with a mixture of pooled sera obtained from chickens immune to leukosis and presumably cell-free tumor extract, kept at 37° during 1 hour. They all developed sarcoma and none showed leukotic blood changes; only one (No. 266) was examined microscopically. The first two subpassages made from this chicken produced sarcoma only, but in the third subpassage erythroleukosis appeared again.

The immune sera were heated to 56°C. during 40 minutes. The proportions of serum and extract varied, and the chicken from which subpassages were made (No. 266) received a mixture containing 0.5 cc. of serum, 0.5 cc. of tumor extract, and 1 drop of fresh normal chicken serum. The remaining chickens received mixtures of serum and tumor extract in various proportions, the extremes of which were 0.1 cc. virus plus 0.9 cc. serum and 0.9 cc. virus plus 0.1 cc. serum.

In a third experiment (Passage XI b) an extract of desiccated tumor tissue was mixed with an equal volume of pooled, inactivated immune sera and incubated during 2 hours at 37°C. This mixture was injected intramuscularly into five chickens without ill effect, whereas all of the five control chickens injected with tumor extract alone developed sarcoma or erythroleukosis. The four immune chickens whose blood was used in this experiment were reinjected with the same tumor extract, and remained healthy.

These experiments suggest that the sera of chickens immune to leukosis have an inhibiting or neutralizing effect on the virus of Strain 13 as concerns its ability to produce tumors as well as leukosis. Neutralization is not always complete, however.

Transmission of Sarcoma 13 with Material Free from Living Cells
Desiccation.—Most, but not all, viruses of sarcoma and of leukosis are resistant to desiccation (1). It seemed possible that Strain 13 is caused by two viruses that differ in resistance to desiccation and that this procedure might separate them. But it was found that dried blood and tumor tissue of Sarcoma 13 produced both sarcoma and

erythroleukosis. Five of six dried samples tested produced tumors in all injected chickens; one sample was inactive.

Donor	Dried material	No. injected	No. of successful injections
3971	Tumor	3	3
3116	Tumor	3	3
4078	Tumor	3	3
4304	Blood	4	0
3014	Blood and spleen	7	7
3116	Blood	3	3

The desiccation in these experiments was done in the frozen state, as previously described (2). In one experiment tumor tissue was also dried *in vacuo* at room temperature. There was no significant difference between the tissues dried *in vacuo* in the frozen state and those dried at room temperature.

	Weight of fresh material	Loss of weight	Result of inoculation
	mg.	per cent	
Dried in the frozen state			
40 min. in desiccator.....	0.946	28.5	Successful
1½ hrs. in desiccator.....	1.001	60.0	Unsuccessful
19 hrs. in desiccator.....	1.072	85.8	Successful
7 days in desiccator.....	—	85.8	Successful
Dried in the unfrozen state			
40 min. in desiccator.....	1.048	67.8	Successful
19 hrs. in desiccator.....	0.866	85.8	Successful

The foregoing experiment was undertaken for two reasons: (a) There are no data available that compare the results of drying oncogenic viruses in the frozen and unfrozen state. (b) It was thought that if the virus is living, there might be an optimum degree of dehydration beyond which it might perish. Dehydration, however, was complete under the conditions of the experiment within 19 hours *in vacuo*; when phosphorous pentoxide was renewed after this time and the material was kept in high vacuum for an additional 6 days, there was

no further loss of weight or of activity. From the chemical standpoint the materials thus dried are probably not water-free.

Freezing of tissue from Sarcoma 13 was done by submerging the minced tumor in alcohol chilled to -30°C . during 30 minutes in a sealed test tube. Frozen and unfrozen material was tested on the same three chickens and there was no significant difference in the rate of development of tumor from the fresh and from the frozen material.

DISCUSSION

The evidence here presented supports the view that a single oncogenic virus may stimulate two different types of cells to seemingly neoplastic growth. This possibility has been suggested by several recent investigators (5, 7, 2) but abundant contradictory data have accumulated in the 2 decades since Rous and his associates first described viruses causing chicken sarcomata of various types. Several viruses with well defined individual characteristics have been described and none of them produced leukosis (1).

The strain here described produces sarcoma (endothelioma) and erythroleukosis. It originated in a chicken injected with leukosis Strain 1, which produces erythroleukosis and, less often, myeloblastic leukemia. Did Strain 13 arise from leukosis Strain 1 as a result of a "mutation" as suggested by Oberling and Guérin (5), or did Strain 1 become contaminated with a virus of a sarcoma that affects endothelial cells of the blood-forming organs? The characteristics of Strain 1 remained unchanged during 5 years of animal passages with the possible exceptional modification described here. Strain 13 also has remained constant during $1\frac{1}{2}$ years of studies in which approximately 300 chickens have been used.

The experiments of McIntosh already quoted (4) may be taken to indicate that tumor-producing viruses are common among chickens. We found them among the experimental birds as well as among the uninjected controls. The possibility that Strain 13 consists of a mixture of two viruses seems, therefore, very likely, but the tests thus far applied have failed to separate them. However, a sarcoma agent that stimulates endothelial cells of the blood-forming organs of almost every young chicken inoculated, and discharges a virus into the blood in high concentration, is obviously not easily separable from a leukosis

agent that stimulates primitive blood cells but otherwise behaves like the sarcoma agent.

Several factors are necessary for a virus to produce manifest disease. The number of disease-producing units in the new host must exceed a certain minimum. This is the probable explanation of the incubation period of leukosis, during which anatomical changes are not detected. It may be supposed that after intramuscular inoculation of Strain 13, the hypothetical sarcoma virus undergoes rapid multiplication and leads to the death of the animal before the leukosis virus reaches a concentration necessary to produce manifest disease. In the subpassages of sarcoma both viruses are carried over into the new host but become manifest only under conditions that allow them to multiply and reach the concentration that would produce disease. This assumption is contradicted by the experiment that showed that a minute amount of the sarcoma tissue (0.00001 cc.) of an apparently leukosis-free chicken produced erythroleukosis only, whereas larger amounts produced sarcoma. It evidently requires a larger dose or a longer period of time to produce sarcoma in the breast muscle than it does to cause diffuse sarcomatosis and erythroleukosis of internal organs, such as spleen and bone marrow.

The observations suggest the possibility that the virus of Strain 13 produces sarcoma primarily and that erythroleukosis is the result of profuse secondary sarcomatosis of the bone marrow. Against this view are the histological characteristics of erythroleukosis that are indicative of a primary proliferative process. There was no difference in this regard between Strain 1 and Strain 13. Attempts to induce erythroleukosis by bleeding or chemicals that injure red cells have not been wholly successful, but pyrocin produced erythroblastic hyperplasia of the marrow resembling closely that produced by the leukosis viruses (3). Erythroleukosis unassociated with sarcoma was seen in very few chickens and in these cases only a small piece of marrow was examined. Sarcoma of the breast muscles may exist over long periods of time without causing blood changes. Histological examinations showed that the virus of Strain 13 caused a profound derangement of the vascular system with extensive hematoma, often with only scant evidence of malignant proliferation. It may be that this derangement is often the first step in the neoplastic transformation of

endothelium. The relation of this virus to endothelial cells and to primitive erythroblasts, as well as the very nature of erythroleukosis, needs further investigation. Erythroleukosis does not appear to be an etiological entity.

It is evident that the adenocarcinoma of the oviduct which was encountered in a leukosis-immune chicken killed 4 months after intra-venous and intramuscular inoculations with Strain 13 was not produced by it since the three chickens into which the tumor tissue was implanted remained healthy. There is no evidence to support the opinion of Oberling and Guérin that viruses which produce neoplasms possess a high degree of adaptability, for the characteristics of both Strains 1 and 13 have remained constant during the past 18 months.

Tissue culture studies offer a less expensive and more promising procedure to determine whether a single virus is capable of producing both sarcoma and leukosis. Such studies have shown (14) that sarcoma cells of Strain 13 incubated at 38°C. retain the ability to produce sarcoma with erythroleukosis during a period of 67 days.

The experiments here described do not warrant definite conclusions regarding the origin and nature of Strain 13. The possibilities considered may be summarized as follows:

1. A single virus known as Strain 13 produces sarcoma (endothelioma) and erythroleukosis. (a) It developed as a variant of the virus of leukosis Strain 1. (b) It occurred spontaneously in a chicken inoculated with Strain 1, but has no etiological relationship to this strain.
2. Strain 13 consists of two viruses, one producing sarcoma (endothelioma), the other (the virus of Strain 1) erythroleukosis. The sarcoma virus occurred independently in a chicken inoculated with the leukosis strain.

SUMMARY

A transmissible strain of sarcoma (Strain 13) is described that took its origin in a chicken inoculated with leukosis of Strain 1. The virus of Strain 13 produces sarcoma in the breast muscle injected with it and after intravenous inoculation it produces diffuse sarcomatosis of the spleen, bone marrow, and several other organs.

The available evidence suggests that Strain 13 is caused by a single virus with ability to produce diffuse endothelial growth in the blood-forming organs associated with extensive hematomata, and erythroblastic proliferation.

The strain can be readily transmitted by material free from live cells and preserved by drying. As small amounts of blood as 0.00001 cc. from chickens with sarcomatosis of the internal organs transmit Strain 13 by the intravenous route.

By implantation sarcoma of Strain 13 can be transferred to chickens that are resistant to repeated inoculations with leukotic viruses.

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EXPLANATION OF PLATES

The photographs of the gross material were made from specimens preserved in Kaiserling solution, and the photomicrographs from sections stained with hematoxylin and azure II-eosin solutions according to the technic of Maximow. The magnifications shown are only approximate.

PLATE 24

FIG. 1. Sarcoma produced in the breast muscle by intramuscular injection of tumor tissue of Strain 13.

endothelium. The relation of this virus to endothelial cells and to primitive erythroblasts, as well as the very nature of erythroleukosis, needs further investigation. Erythroleukosis does not appear to be an etiological entity.

It is evident that the adenocarcinoma of the oviduct which was encountered in a leukosis-immune chicken killed 4 months after intravenous and intramuscular inoculations with Strain 13 was not produced by it since the three chickens into which the tumor tissue was implanted remained healthy. There is no evidence to support the opinion of Oberling and Guérin that viruses which produce neoplasms possess a high degree of adaptability, for the characteristics of both Strains 1 and 13 have remained constant during the past 18 months.

Tissue culture studies offer a less expensive and more promising procedure to determine whether a single virus is capable of producing both sarcoma and leukosis. Such studies have shown (14) that sarcoma cells of Strain 13 incubated at 38°C. retain the ability to produce sarcoma with erythroleukosis during a period of 67 days.

The experiments here described do not warrant definite conclusions regarding the origin and nature of Strain 13. The possibilities considered may be summarized as follows:

1. A single virus known as Strain 13 produces sarcoma (endothelioma) and erythroleukosis. (a) It developed as a variant of the virus of leukosis Strain 1. (b) It occurred spontaneously in a chicken inoculated with Strain 1, but has no etiological relationship to this strain.
2. Strain 13 consists of two viruses, one producing sarcoma (endothelioma), the other (the virus of Strain 1) erythroleukosis. The sarcoma virus occurred independently in a chicken inoculated with the leukosis strain.

SUMMARY

A transmissible strain of sarcoma (Strain 13) is described that took its origin in a chicken inoculated with leukosis of Strain 1. The virus of Strain 13 produces sarcoma in the breast muscle injected with it and after intravenous inoculation it produces diffuse sarcomatosis of the spleen, bone marrow, and several other organs.

The available evidence suggests that Strain 13 is caused by a single virus with ability to produce diffuse endothelial growth in the blood-forming organs associated with extensive hematomata, and erythroblastic proliferation.

The strain can be readily transmitted by material free from live cells and preserved by drying. As small amounts of blood as 0.00001 cc. from chickens with sarcomatosis of the internal organs transmit Strain 13 by the intravenous route.

By implantation sarcoma of Strain 13 can be transferred to chickens that are resistant to repeated inoculations with leukotic viruses.

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EXPLANATION OF PLATES

The photographs of the gross material were made from specimens preserved in Kaiserling solution, and the photomicrographs from sections stained with hematoxylin and azure II-eosin solutions according to the technic of Maximow. The magnifications shown are only approximate.

PLATE 24

FIG. 1. Sarcoma produced in the breast muscle by intramuscular injection of tumor tissue of Strain 13.

LEUKOSIS, AND SARCOMA OF CHICKENS. I

FIG. 2. Diffuse sarcomatosis of the spleen (*S*) contrasted with erythroleukosis (*E*) of that organ. Sarcomatosis causes slight enlargement of the spleen, increased consistency, and grey-white discoloration. Erythroleukosis causes moderate or great enlargement of the spleen and grey-red discoloration.

FIG. 3. Hematomata in the spleen and liver produced by proliferation of endothelial cells, with disruption of blood vessels. The immediate cause of death was hemorrhage from the liver.

PLATE 25

FIG. 4. Sarcomatosis (*S*) Strain 13 of the bone marrow contrasted with erythroleukosis (*E*). In the marrow of chickens with sarcomatosis there are solid tumor masses (*t*) filling the cavity of the marrow and infiltrating the cortex of the bone; cysts (*c*), and hemorrhages (*h*). In erythroleukosis the marrow is uniformly grey-red.

FIG. 5. Bone marrow in Sarcoma 13 showing cavities lined with endothelial cells and filled with serum and nucleated erythrocytes. $\times 75$.

FIG. 6. Bone marrow in Sarcoma 13 showing dense masses of spindle-shaped tumor cells. $\times 350$.

PLATE 26

FIG. 7. Mucinous degeneration with formation of cavities in sarcoma of the breast muscle. Azure II stains this mucinous substance blue. $\times 75$.

FIG. 8. Shows the predominant type of cell of Sarcoma 13 produced by intramuscular inoculation. There are mitotic figures. $\times 650$.

FIG. 9. Multinuclear giant cells in sarcoma of the breast muscle, produced by Strain 13. $\times 650$.

FIG. 10. Section from a testis showing cavities lined by endothelial cells and filled with erythroblasts and erythrocytes. $\times 400$.

PLATE 27

FIG. 11. Diffuse sarcomatosis of the spleen with replacement of lymphoid tissue. The dilated sinuses are filled with erythroblasts and erythrocytes. $\times 350$. The gross appearance of such a spleen is shown in Fig. 2.

FIG. 12. Malignant proliferation of endothelial cells narrowing the lumen of a blood vessel in the spleen. The vessel is filled with erythroblasts and erythrocytes and there is normal lymphoid tissue about the vessel. $\times 350$.

FIGS. 13 and 14. Diffuse sarcomatosis of the ovary. The neoplastic endothelial cells form sinusoidal channels filled with erythroblasts. Magnifications: Fig. 13 $\times 130$; Fig. 14 $\times 400$.

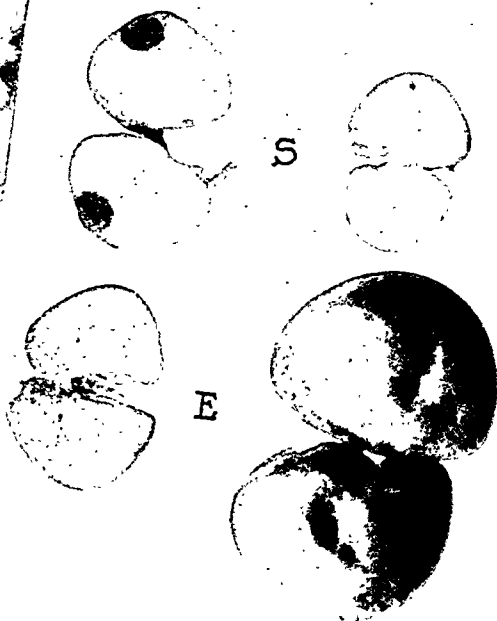
PLATE 28

FIG. 15. Cavernous endothelioma of the skin containing erythrocytes in variable numbers. $\times 65$.

FIG. 16. Shows another microscopic field of the same endothelioma. The cavities are smaller and there is solid tumor tissue in the lower part of the field. $\times 65$.

FIG. 17. Higher magnification of the same endothelioma of the skin, showing the endovascular formation of tortuous blood channels arising seemingly from the endothelial cells lining the cavities. $\times 250$.

FIG. 18. Diffuse hypertrophy and hyperplasia of endothelium of the liver, probably neoplastic. The capillaries are greatly distended with blood cells, and the liver cells are atrophied. The arrows point to remnants of liver cells. $\times 300$.

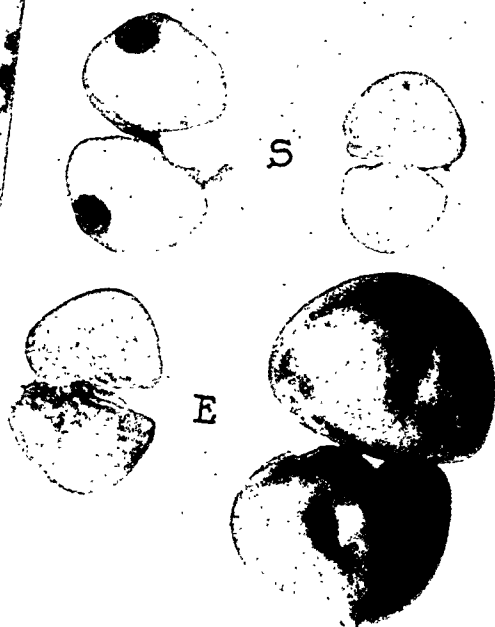


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Continued from Plate 23, Figure 1.

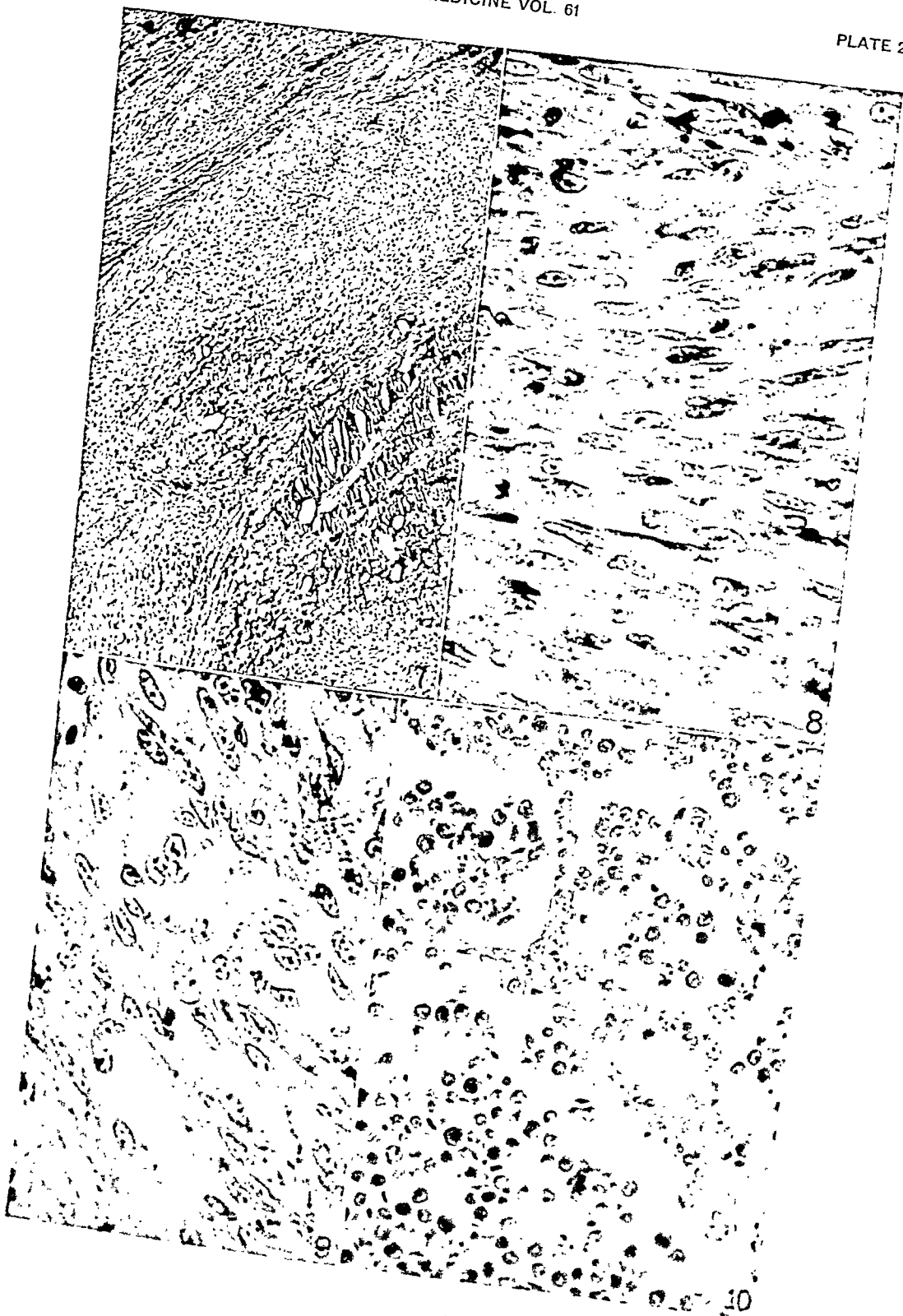


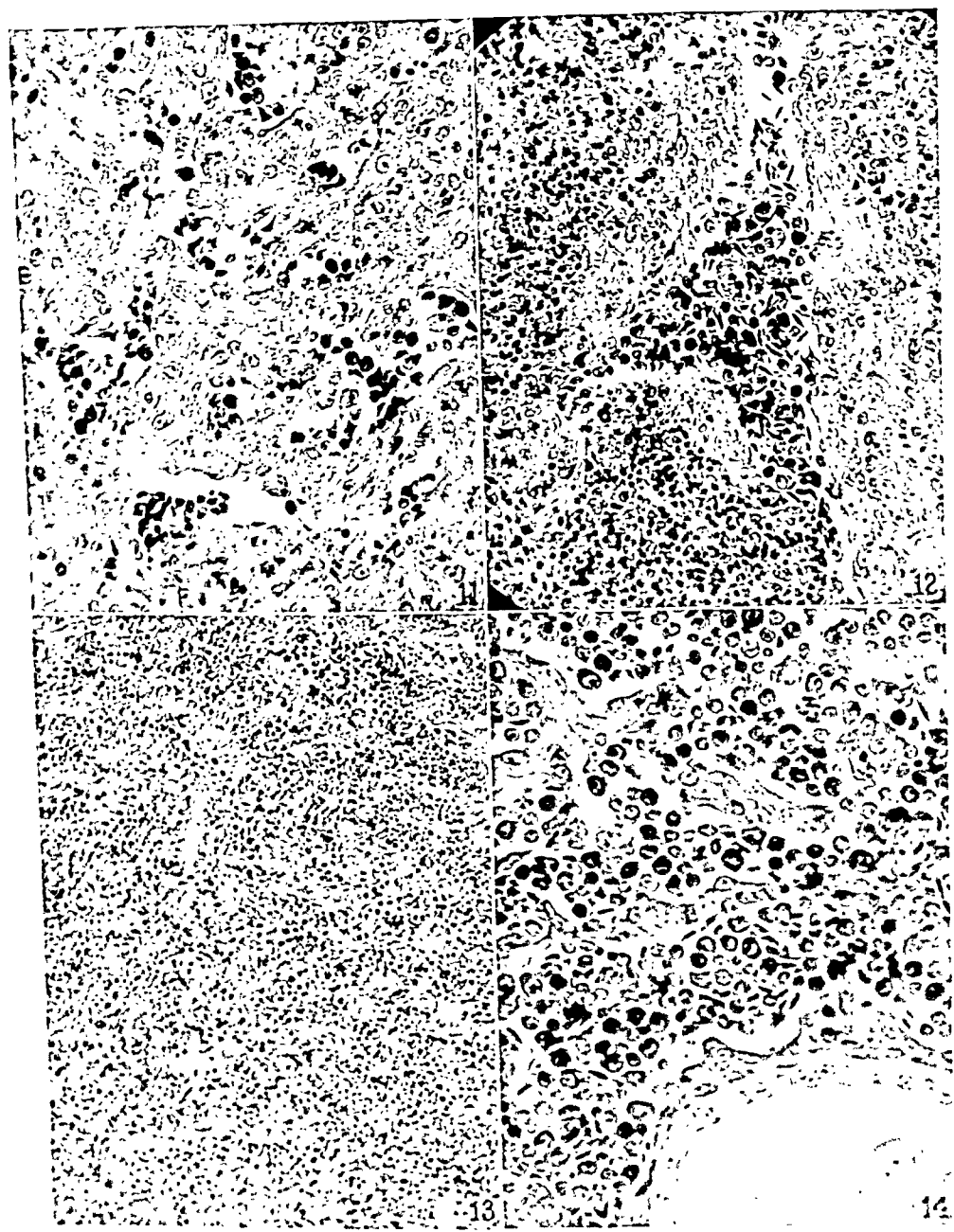
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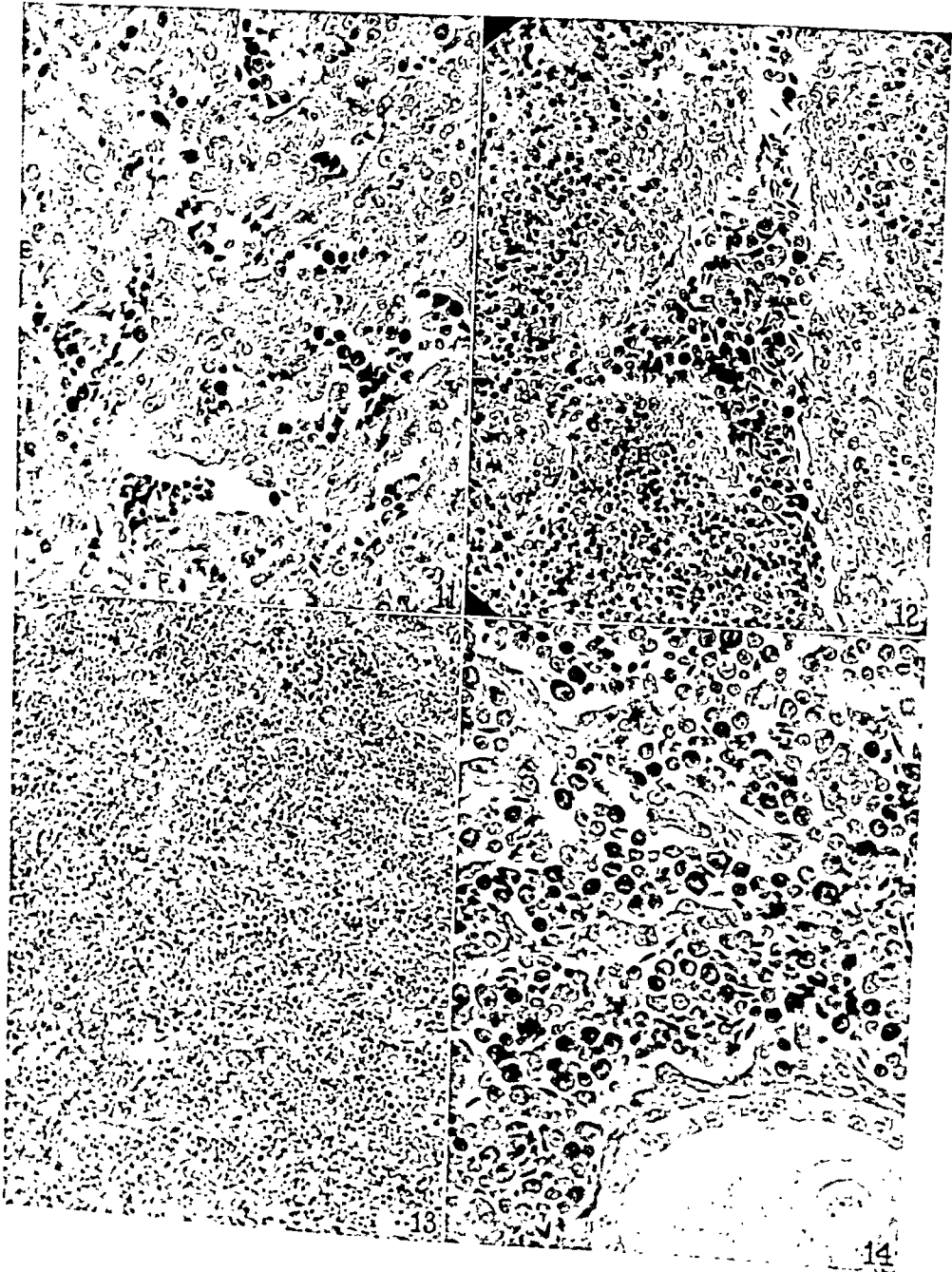
3

Crystals and Fungus, Leukemia, and sarcoma of children. D





SECTION 11. (11) (12) (13) (14)



(Stubbs and Furth: Leukosis, and sarcoma of chickens. D)



(Stubbs and Furth. Leukemia, and sarcoma of children. 11)

THE EXTENT OF LOCAL DISPERSION OF INFECTIOUS AGENTS AS A FACTOR IN RESISTANCE TO INFECTION

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PLATE 29

(Received for publication, October 27, 1934)

The reactions of the host to bacterial and virus infections have been measured in terms of the minimal lethal dose for different sites of inoculations, and they have been appraised in terms of damage to tissues and in terms of tissue specificity; but the extent of dispersion of the infective agents as affecting the results has in the past been accorded little attention. In recent work from this laboratory a chemical fraction which causes dispersion of particulate matter injected with it into the skin and extracts from testicle (1) and from invasive bacteria (2), which have the same effect, served as a tool to show that the extent of dispersion of the infectious agent importantly influences the size of the lesions due to infection. It was found that intracutaneous inoculations into rabbits of testicle extract together with concentrated suspensions of bacteria resulted in more widespread lesions than inoculations of equally concentrated suspensions of bacteria in isotonic saline (3). It was later observed that inoculations of testicle extract with dilute suspensions of staphylococcus either produced lesions which were less widespread than those produced by saline suspensions or failed to produce a lesion (4). These results led to the supposition that in the case of bacteria there could be determined a critical concentration, the minimal effective concentration per unit area of infection, below which no reaction would occur, and above which the intensity of the reaction would be roughly proportional to the concentration. The critical concentration is presumably the resultant of local resistance of the host tissues, and of local virulence of the bacteria.

This supposition has now been tested repeatedly with bacteria and with several viruses. The results to be given in the present paper confirm and extend those previously reported, as concerns bacteria. They indicate in addition that the critical concentration for filterable viruses may depend upon a different state of affairs from that for bacteria.

Material and Methods

Origin and Characteristics of the Bacteria Used.—*Staphylococcus albus*.—This was an R variant, viscid and non-virulent, isolated 2 years ago from a chronic suppurative process in man.

Staphylococcus "citreus".—This was a hyperpigmented, viscid, non-virulent strain obtained by dissociation of a virulent *aureus* strain growing in gelatin medium.

B. prodigiosus, E. typhi, V. cholerae, B. proteus, B. dysenteriae Flexner.—The sources of these are not accurately known, but they are all old laboratory strains of low virulence. The strain of *B. prodigiosus* occasionally shows a rather strong local infective power for certain rabbits.

Pneumococcus, R Variant.—This was the Strain 1/192/R, obtained by dissociation of a virulent strain of *Pneumococcus* Type I, and is non-virulent for mice and rabbits.¹

Streptococcus 090 4/8 Hemolytic.²—This is a bovine strain which is highly virulent for mice and rabbits.

Streptococcus 090 4/9 Non-Hemolytic.—This is also a bovine strain and highly virulent for mice, but probably less so for rabbits. Both of these strains of streptococcus contain the specific carbohydrate and are very active producers of the spreading factor.

Streptococcus S/43-M.A.—Isolated from a case of measles, it is non-virulent for mice and of medium virulence for rabbits. It contains the M. substance and produces a moderate amount of spreading factor.

Streptococcus S/43 Glossy.—This was derived from Strain S/43-M.A. with which it shares its characteristic of virulence. It is devoid of type-specific substance and is a very feeble producer of spreading factor.

Streptococcus K-152.—A rheumatic strain, it is probably of medium virulence for rabbits.

Staphylococcus aureus, Strains 40 and 50.—These are virulent strains isolated

¹ This strain was supplied by Dr. Goodner of the Hospital of The Rockefeller Institute.

² All of these strains of streptococcus were supplied by Dr. Lancefield of the Hospital of The Rockefeller Institute. The nomenclature used by her to designate these strains has been retained.

2 years ago from an abscess in man. Both are very active in production of the spreading factor.

Bacillus of Mouse Typhoid I and II.—These strains are closely related to or identical with *B. enteritidis* and *B. pestis caviae* respectively. They were isolated from spontaneous cases in mice and are very virulent for these animals and for rabbits.³

Bacillus of Mouse Typhoid III.—A strain highly virulent for mice and rabbits, it was isolated from a spontaneous case in this laboratory during an epidemic.

Pneumococcus Type I.—This was the Neufeld strain, very virulent for both mice and rabbits. It is reported to kill a mouse when doses as low as 1 to 3 bacteria are injected.⁴

All of the above described strains have been kept in artificial media for several years.

Preparation of Suspensions of Bacteria.—24 hour and occasionally 48 hour cultures of bacteria on plain or blood agar were chosen for the tests, save in the case of pneumococcus which was grown in T broth containing rabbit blood. Homogeneous concentrated suspensions of living bacteria were prepared by shaking the cultures with isotonic saline in tubes containing beads. The addition of different volumes of the isotonic saline to aliquots of the concentrated bacterial suspension provided a graded series of dilutions of bacteria. The most dilute suspension tested was 1/2,000 millionth of a 24 hour culture; the most concentrated was 1/200th of a 24 hour culture.

Origin and Characteristics of the Filterable Viruses Used.—*Neurovaccine Virus.*—This is the well known strain of Levaditi which has been carried through rabbit brain for many years. It is particularly virulent for rabbits, especially when inoculated intracerebrally.

Testicle Vaccine Virus.—This is the Noguchi strain, carried for many years through rabbit testicle. It is moderately active for rabbits when injected into the testicle or skin, but does not kill after intracerebral injection.

Dermovaccine Virus.—This is in the form of plain lymph from the infected cow, prepared by the New York City Board of Health. It is moderately active for rabbit skin.

Cultured Vaccine Virus.—This is the same as the last mentioned, kept in Rivers' medium for 80 passages (6).⁵

Virus III.—This virus was discovered by Dr. Rivers in rabbit testicle and carried through this organ for several years. With moderate activity for rabbit testicle, it gives only very mild lesions when injected into the skin.

Virus of the Fibroma-Like Growth of Rabbits.—This virus was discovered by Dr.

³ These strains were supplied to us by Dr. Webster of The Rockefeller Institute (5).

⁴ This strain was supplied to us by Dr. Goodner.

⁵ This strain was supplied by Dr. Rivers.

Shope in wild rabbits (7).⁶ It was carried by us through 18 passages in the rabbit skin, after which it induced much more active growths than before. It was active only when injected into the skin or testicle, producing a strictly local growth which always regressed.

Preparation of Suspensions of Viruses.—Graded series of dilutions of virus were prepared from standard suspensions of the virus contained in the tissue pulp.

The standard suspensions of Levaditi's neurovaccine virus and of Noguchi's testicle vaccine virus were prepared as follows. Rabbits injected intratesticularly with the virus were killed 5 days later, the testes were removed and ground with sand and approximately 10 volumes of a mixture of Ringer's solution and glycerine in equal parts. The resulting pulp was distributed in tubes, covered with a layer of vaseline, and stored in the ice box. Dilutions of this pulp were made up immediately before each experiment.

For the preparation of Rivers' Virus III the rabbits were similarly treated, but the testicle pulp containing the virus was used immediately upon preparation. The same graded dilutions were prepared from the original pulp of the plain and cultured vaccine virus.

The virus of Shope's fibroma-like growth was used after 2 passages and after 18 passages through the rabbit skin. Growths from 5 to 8 days old were used. The fresh tissue was ground with sand and 6 volumes of isotonic saline; the resultant pulp was centrifuged and the supernatant fluid used for dilutions.

Preparation of the Spreading Agents.—Bull testicle extract was prepared by stripping the membrane from the organ and grinding the glandular tissue with sand and 4 volumes of isotonic saline. The extract was then filtered through a Berkefeld V or W filter.

The bacterial spreading factor was prepared from an invasive strain of staphylococcus (2) by extracting a 24 hour culture of the bacteria for about 15 minutes with isotonic saline in the ratio of 5 or 10 cc. of fluid per culture and then filtered through a Berkefeld V candle. Both testicle and staphylococcus extracts stored in tubes in the ice box were still active several months after their preparation.

Immune Sera.—The antipneumococcus serum was either the horse serum from the New York Board of Health or the rabbit antiserum prepared according to the method of Cole and More (8). The antivaccine sera came from 3 rabbits which had recovered from vaccine infection of the skin and are designated as Samples A, B, and C. They had been previously tested for antivaccinal antibodies.

Method of Testing the Effects of Spreading of Infectious Agents.—Progressive dilutions of the infectious agent to be tested were made with saline solution. Generally no more than 6 of such dilutions were tested on a single rabbit. 1 cc. of a mixture of equal volumes of each dilution with testicle extract was injected intracutaneously into the side of the animal, and these injections were repeated in the other side with saline instead of testicle extract. Experiments performed in this way exclude the influence of individual variations in animal resistance upon

⁶ The virus was supplied to us by Dr. Shope.

the lesions produced by the different dilutions of the infectious agent. In certain series of experiments each of the injections was made in a single rabbit, for reasons which will be explained later.

The lesions are described in terms of the area of skin affected and also of the intensity of the various signs of inflammation. These manifestations of infection were recorded daily by plus signs for 7 or more days after the inoculations. Lilac rabbits of the breed of The Rockefeller Institute have been used throughout.

Preliminary Considerations on the Uniformity of Dispersion of Infective Agents in the Skin When Injected Together with Testicle Extract

All the data so far available on the effect upon local infections of the spreading factors from testicle extract (1), extracts of other organs (9), and of invasive bacteria (2) and azoproteins (10) point directly to the following explanation: The modification of the lesion is due to an induced increase of permeability of the tissue. As a consequence of this the infective agent injected along with the spreading factor is passively distributed throughout a larger area of tissue than ordinarily.

Previous work has shown (1) that when a suspension of colored matter such as India ink or Prussian blue is injected in the rabbit skin the mixture seems to distribute itself rather uniformly, as judged by the density of the blackening to the naked eye 1 or 2 hours after injection. Nevertheless, the histological study of the injected area shows a gradual thinning out toward the edges and moreover it shows a clustered distribution of the injected particles, this latter suggesting that a flocculation out of suspension has taken place in the living tissue.⁷

As a preliminary step in our work we have studied the distribution of bacteria in the subcutaneous tissue shortly after the suspensions of infective agents were injected into the rabbit skin together with testicle extract. 2 hours were permitted to elapse before the animals were killed, so as to allow a spreading of the mixture to take place. It is realized that some bacterial multiplication may have occurred during this time, but that was a matter of no moment, since in the present study we are particularly concerned with the lesions resulting from the multiplication of bacteria after their lodgement.

⁷ When spreading preparations more active than those used in the present work are injected together with India ink the spreading steadily increases for several hours after inoculation. In such cases the distribution of the injected particles is uneven, as attested by the irregular darkening of the skin.

Test.—Homogeneous suspensions in saline of 24 hour cultures of *E. coli*, *B. prodigiosus*, and *Streptococcus hemolyticus* 090 were prepared. A 1/10,000th of the *E. coli* and *B. prodigiosus* cultures and a 1/1,000th of the streptococcus culture were each suspended in 0.5 cc. of saline, mixed with an equal volume of testicle extract, and injected into an area of the depilated skin of the same rabbit. The animal was killed after 2 hours. By that time each of the injected mixtures had spread through an area of approximately 6.5 x 5.5 cm. from the point of inoculation, as shown by local edema and blanching. With sterile instruments circles of skin about 0.3 cm. in diameter were then excised from near the point of inoculation and from other regions inside and just outside the limits of the affected skin. The fragments of skin were firmly held with forceps to express the fluids and the under sides of each were rubbed over the surface of blood agar plates. The number of colonies which grew from each of the fragments after 48 hours of incubation at 37°C. are given in Table I.

TABLE I

Number of Bacteria Distributed at and around the Point of Intradermal Inoculation of Bacterial Cultures plus Testicle Extract

	Point of inoculation	1-3 cm.	4-6 cm.	7-8 cm. outside area of spreading
<i>B. prodigiosus</i>	360	300	300	0
<i>E. coli</i>	500	400	600	0
<i>Streptococcus hemolyticus</i> 090.....	70	400	300	35

Table I shows that in the case of *B. prodigiosus* and *E. coli* the number of bacteria within the area of skin affected by the spreading is remarkably uniform, if one judges by the number of colonies which have developed from the different regions of skin as result of the relatively crude procedure. In the case of *Streptococcus hemolyticus* 090 a spread of the bacteria from the area of inoculation and beyond the visible limit of dermal infiltration with fluid evidently took place. This strain of streptococcus is itself a very active producer of the spreading factor (2), which may perhaps account for the findings. The results as a whole are constant enough to permit of the conclusion that to all intents and purposes the distribution in the infiltrated skin of bacteria shortly after injection of them together with testicle extract is at least fairly uniform. The demonstration that there is some uniformity proved of value in interpreting the phenomena that were studied later.

The Effect of Spreading upon Bacteria of Low Virulence

The first tests on the effect of varying degrees of bacterial dispersion through the tissues were carried out with a group of organisms of low virulence for rabbits.

Experiment.—Each strain of bacterium was tested by intradermal injection in several dilutions with testicle extract on one flank of a rabbit and on the other of the same dilutions with normal salt solution. Each test was repeated on 2 or 3 animals. The bacterium used, the range of dilutions, and the extent of the lesions as determined in a total of 19 rabbits are given in Table II.

Table II shows definitely that when the number of bacteria is still sufficient to cause a lesion in the control inoculations, this same number, spread through a larger area of skin under the influence of testicle extract, gave rise to no lesions or lesions of lower intensity. The latter were somewhat difficult to classify on account of their diffuse character.⁸

Effect of Spreading upon Bacteria of Moderate to High Virulence

The strains of bacteria used for these experiments were of moderate or high virulence.

Experiment.—As in the previous test, each strain was injected with and without testicle extract in the skin of the same rabbit, each test being repeated from 2 to 4 times. Results based on 24 rabbits inoculated with the various bacteria are given in Table III.

It is clear from Table III that above a concentration of bacteria, which may be termed the critical one, dispersion by testicle extract results in enhancement of the lesion, while at or below this concentration dispersion results in the diminution of any local manifestation of infection. All the strains tested showed this reversal of the effect of testicle extract, except *Pneumococcus* Type I, an exception which will be dealt with later. It will be noted in Tables II and III that *B. dysenteriae* Flexner and the bacillus of mouse typhoid produce a pustular lesion when injected intracutaneously in low concentration

⁸ It may well be said here that several tests carried out in order to detect a possible bactericidal effect of the testicle extract on bacteria *in vitro* always gave negative results.

TABLE II

*The Lesions Produced by Bacteria of Low Virulence, Injected with Saline and Testicle Extract Respectively
(Recorded at the Time of the Maximum Lesion, 2 to 6 Days after Injection)*

Dilutions of bacterial suspensions of 24 hr. agar or broth culture)	<i>Staphylococcus citreus</i> *		<i>Staphylococcus albus</i> *		<i>B. prodigiosus</i> *		<i>E. coli</i>		<i>E. typhi</i> *		<i>V. cholerae</i> *		<i>B. proteus</i>		<i>B. dysenteriae Flexner</i>		<i>Pneumococcus R form</i>	
	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract
1/200	1+	-	2+	±	2+	±	3+	±	2+	±	2+	2+	3+	±	2+	1+	1+	-
1/1,000	1+	-	2+	±	1+	-	2+	±	-	-	-	-	2+	±	±	±	±	-
1/2,000	1+	-	1+	-	1+	-	1+	-	2+	-	3+	±	2+	-	±	-	-	-
1/10,000	±	-	1+	-	1+	1+	±	-	-	-	±	±	±	1+	1+	-	-	-
1/20,000	-	-	1+	-	±	-	±	-	±	-	-	±	±	1+	1+	-	-	-
1/200,000	-	-	±	-	-	-	-	-	-	-	-	-	-	1+	1+	-	-	-
No. of tests	2		2		2		2		2		2		2		2		3	

p refers to lesions consisting of discrete pustules.

* Other tests have shown that more concentrated suspensions give enhanced lesions under the influence of testicle extract or of the spreading factor from invasive staphylococcus (2).

TABLE III
Lesions Produced by Bacteria of Moderate or High Virulence, Injected with Saline and Testicle Extract Respectively

(Recorded at the Time of the Maximum Lesion, 2 to 6 Days after Injection)

Dilutions of bacterial suspensions (duration of 24 hr. near culture)	Streptococcus 090 non- hemolytic*		Streptococcus S/43 M.A.		Streptococcus hemolyticus 090*		Staphylococcus aureus (Strain 40)		Bacillus of Mouse Typhoid I		Bacillus of Mouse Typhoid II		Bacillus of Mouse Typhoid III		Pneumococcus Type I	
	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract
1/200	3+	—	5+	7+	8+	8+	6+	10+	5+	10+	5+	10+	4+	9+	4+	11+
1/1,000	2+	—	5+	4+	6+	4+	4+	9+	4+	9+	4+	9+	4+	9+	4+	6+
1/2,000	1+	—	4+	2+	6+	±	3+	7+	4+	7+	4+	7+	4+	7+	3+	6+
1/10,000	1+	—	3+	2+	5+	1+	3+	5+	3+	6+	3+	5+	3+	5+	2+	6+
1/200,000	±	—	3+	1+	5+	1+	3+	3+	3+	3+	5+	2+	3+	5+	±	5+
1/2 × 10 ⁶	—	—	3+	1+	3+	—	3+	3+	3+	3+	5+	2+	3+	5+	1+	4+
1/10 ⁷	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/2 × 10 ⁷	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/4 × 10 ⁷	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/10 ⁸	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/2 × 10 ⁸	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/4 × 10 ⁸	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/8 × 10 ⁸	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/16 × 10 ⁸	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/10 ⁹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/2 × 10 ¹⁰	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
No. of tests, . . .	3	3	3	4	2	3	2	3	2	3	2	3	3	4		

p refers to lesions consisting of discrete pustules.
Arrows indicate minimal effective concentration of bacteria.

* Other tests have shown that more concentrated suspensions give enhanced lesions under the influence of testicle extract.

with testicle extract. These pustules resemble those produced by some filterable viruses. They do not appear until 5 to 7 days after inoculation and it is believed that they are manifestations of the gradual multiplication of a few scattered organisms. Such lesions are illustrated in Figs. 1, 2, 3, and 4.⁹

The Effect of Spreading upon Pneumococcus Type I Lesions When the Host's Resistance Is Raised

Reference has been made to the fact that virulent pneumococcus was enhanced even in high dilutions by testicle extract, in this respect differing from the other bacteria tested. Non-virulent strains of this organism do not show this characteristic, as is evident from Table II. It seems not unlikely that the great susceptibility of the rabbit to the virulent organism might account for the difference observed. The following experiment was undertaken with the aim of testing the effect of an increase in the animal's resistance upon the lesions produced by the virulent organism disseminated by testicle extract.¹⁰

Experiment.—8 rabbits weighing approximately 2,500 gm.¹¹ were injected intracutaneously on one flank with graded dilutions of an 18 hour T broth culture of *Pneumococcus* Type I mixed with testicle extract. On the other flank they received a series of the same graded dilutions of the organism but in salt solution. Immediately after the inoculations 2 of the rabbits received 5 cc. each of antipneumococcus horse serum, another 2 cc., and a 4th 1 cc. The 5th animal was given 5 cc. of antipneumococcus rabbit serum and the 6th 5 cc. of normal horse serum. The remaining 2 received no serum and served as controls. The results are given in Table IV.

Additional tests were made on 3 rabbits in which graded dilutions of the organisms were mixed with testicle extract and antipneumococcus serum, and the

⁹ The photographs in another paper (3) illustrate further the effect of spreading to suppress lesions produced by *Staphylococcus albus* and *aureus*.

¹⁰ These experiments were suggested in part by an observation on 2 strains of streptococcus. When we studied these strains (S/43 glossy and K-152) by the technique of spreading their dilutions, each strain gave 2 sets of opposite but perfectly consistent results. In certain rabbits, enhancement of the lesions produced by high dilutions of the bacterial suspension was obtained; on other rabbits, suppression of the lesions produced by low dilutions was observed. It seemed evident that the degree of individual immunity might have something to do with the results.

¹¹ Smaller animals are not well suited for this kind of experiment.

TABLE IV
*Lesions Produced by Progressive Dilutions of Pneumococcus Type I Culture Injected with Testicle Extract and Isotonic Saline
 Respectively in the Rabbit Partly Immunized by the Specific Antiserum (Lesions Recorded after 24 Hours)*

Rabbit No.	Lesions produced by pneumococcus culture plus isotonic saline (fractions of an 18 hr. broth culture)															Serum injected in vein	General result
	1/1,000,000																
	1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000	1/100,000,000	1/1,000,000,000	1/10,000,000,000	1/100,000,000,000	1/1,000,000,000,000	1/10,000,000,000,000	1/100,000,000,000,000	1/1,000,000,000,000,000		
1	-	-	-	-	-	-	-	-	7+	5+	2+	1+	1+	-	-	5 cc. antipneumo- coccus horse serum	Survived
2	±	-	-	-	-	-	-	-	5+	3+	2+	1+	±	-	-	5 cc. antipneumo- coccus horse serum	Survived
3	1+	-	-	-	-	-	-	-	6+	6+	1+	-	-	-	-	2 cc. antipneumo- coccus horse serum	Survived
4	1+	-	-	-	-	-	-	-	7+	6+	±	-	-	-	-	1 cc. antipneumo- coccus horse serum	Survived
5	±	-	-	-	-	-	-	-	5+	5+	1+	-	-	-	-	5 cc. antipneumo- coccus rabbit serum	Died after 3 days
6	8+	6+	5+	2+	2+	-	-	-	5+	4+	2+	1+	1+	-	-	5 cc. normal horse serum	Died after 5 days
7	8+	6+	6+	5+	4+	3+	2+	2+	5+	3+	2+	1+	1+	1+	1+	No serum injected	Died within 24 hrs.
8	7+	5+	4+	2+	2+	2+	2+	2+	5+	3+	2+	1+	1+	1+	1+	No serum injected	Died within 24 hrs.

mixtures injected in one flank of the rabbit. The control injections consisted of the same series of dilutions with antiserum and saline solution. The amount of antiserum varied from 0.05 cc. to 0.0005 cc., but that of testicle extract and of saline was kept constant. The results are given in Table V.

It is evident from Table IV that a marked inhibition of the pneumococcus infection results when the organisms are spread by testicle extract if the resistance of the animal has been raised by the injection

TABLE V

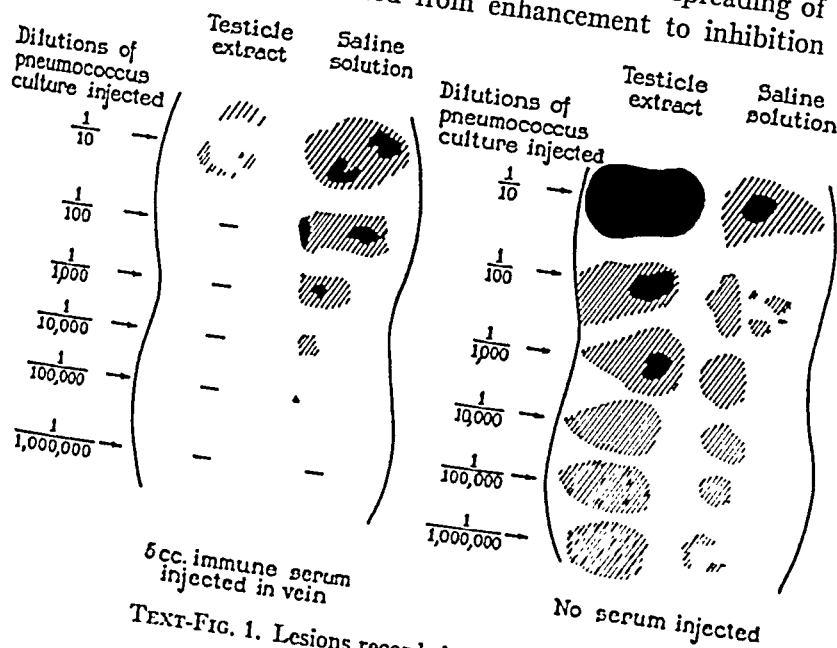
Lesions Produced by Mixtures of Pneumococcus Type I and Specific Antiserum Together with Testicle Extract and Saline Respectively (Lesions Recorded after 24 Hours)

Skin site	Fraction of pneumococcus culture (contained in 0.5 cc.)	Amount of saline solution	Amount of testicle extract	Amount of antipneumococcus serum	Resultant lesion	
		cc.	cc.	cc.		
Rabbit 1	1	1/10	0.5	0	0.05	±
	2	1/10	0.5	0	0.05	±
	3	1/10	0	0.5	0.05	±
	4	1/10	0	0.5	0.05	±
	5	1/10	0.5	0	0	3+
	6	1/10	0	0.5	0	6+
Rabbit 2	1	1/10	0.5	0	0.005	2+
	2	1/10	0	0.5	0.005	1+
	3	1/100	0.5	0	0.005	±
	4	1/100	0	0.5	0.005	—
	5	1/100	0.5	0	0	6+
Rabbit 3	1	1/10	0.5	0	0.0005	5+
	2	1/10	0	0.5	0.0005	3+
	3	1/100	0.5	0	0.0005	3+
	4	1/100	0	0.5	0.0005	±
	5	1/100	0.5	0	0	8+

of antiserum. The evidence of increased resistance of the rabbits is manifest also by the suppression of lesions where the highest dilutions of the controls were injected. In those animals receiving no antiserum all of the dilutions produced a uniform enhancement of the lesions. These results are graphically shown in Text-fig. 1, which is based on Rabbits 2 and 7 of the table.

Mixing the antiserum with the bacteria and testicle extract before inoculation as shown in Table V yields essentially the same consequences but to a much lesser extent. This observation practically eliminates the possibility that the suppression of lesions that was observed in the first group was due to a localization of the antibody by the extract.

The two groups show definitely that the effect of spreading of the pneumococcus can be reversed from enhancement to inhibition by



TEXT-FIG. 1. Lesions recorded after 24 hours.

raising the resistance of the host with specific antiserum. Such results in partially immunized animals are perhaps to be explained on the basis of a reduction of the number of pneumococci per area of lesion to a point below the critical number required for infection under the circumstances of an increased state of resistance of the host.

Effects of the Spreading of Individual Bacterial Dilutions

The technique used throughout this study of testing 6 dilutions of bacteria on the same rabbit had an obvious disadvantage. It was difficult to evaluate the effects of the individual injections, since frequently the animals died too soon after the inoculation of such a

large quantity of bacteria. In order to obviate this disadvantage a series of experiments was conducted with progressive dilutions of bacterial cultures combined with testicle extract or saline solution, each rabbit receiving but a single injection. In this way the evolution of the individual lesions could be watched independently of the general effect of any other inoculation.

The following series of experiments was conducted on *Streptococcus hemolyticus* 090, *Staphylococcus aureus*, and *Staphylococcus albus*.

Streptococcus hemolyticus 090.—7 progressive dilutions of a 48 hour blood agar culture were obtained in saline. These dilutions were such that each 0.5 cc.

TABLE VI
Effect of Dispersion of a Single Test Mixture

Type of bacteria	No. of experiments	Enhancement of lesions by testicle extract	Partial suppression of lesions by testicle extract	Complete suppression of lesions by testicle extract
<i>Streptococcus</i>	11	4	2	5
<i>Staphylococcus aureus</i>	2	0	2	0
<i>Staphylococcus albus</i>	2	0	0	2

15 other rabbits were injected with the same bacterial dilutions plus saline solution as control.

represented the following fractions of the culture: 1/200, 1/2,000, 1/10,000, 1/20,000, 1/40,000, 1/60,000, and 1/100,000.

7 rabbits were injected intradermally with one or another of these culture fractions together with the same volume (0.5 cc.) of testicle extract. 7 more rabbits were similarly injected using saline instead of testicle extract. The tests dealing with some of the culture fractions were repeated 2 or 3 times so that the total number of animals used was 22.

Staphylococcus aureus.—Fractions of 1/500 and 1/1,000 of a 24 hour plain agar culture were used. These were injected with 3 cc. of testicle extract or saline into the skin of 4 rabbits.

Staphylococcus albus.—All was done as in the case of *Staphylococcus aureus*.

The lesions were followed until the death of the animal or until complete healing occurred. The end-results of these 3 series of experiments are summarized in Table VI.

It will be seen from Table VI that the dispersion by testicle extract of single test mixtures results in either enhancement or suppression

of the local lesion, as was the case when varying bacterial dilutions were injected in a single rabbit.

The following supplementary results seem worthy of record.

All rabbits injected with doses of streptococcus stronger than $1/20,000$ of a culture, with or without testicle extract, died within 6 days after injection. Some had their lesions enhanced and others partially inhibited by the spreading as compared with the lesions of the corresponding controls. Rabbits injected with $1/20,000$ of a culture either died or survived according to whether they had their lesions enhanced or suppressed by testicle extract. All rabbits injected with less than $1/20,000$ of a culture survived and all had a complete suppression of the skin infection as result of the bacterial spreading. The control lesions in these cases were mild.

All rabbits injected with *Staphylococcus aureus* or *albus* with or without testicle extract survived. The skin lesions were mild.

As a conclusion from this group of experiments it seems safe to state that, despite the influence of individual variations in host resistance on the results of the group of experiments, they must be considered significant. In certain cases rabbits inoculated with suspensions of bacteria in testicle extract showed few or no signs of local or general infection, whereas those inoculated with suspensions of bacteria in isotonic saline developed large lesions and some of them died.¹² These results are consistent with those recorded in Tables II and III and they reveal that an otherwise lethal dose of bacteria can be rendered harmless by admixture with testicle extract.

The Effect of Spreading on Filterable Viruses

The same general procedures were next applied to the viruses.

Experiments.—Each strain of virus was tested with and without testicle extract on the skin of 1 or more rabbits. The results obtained in 18 animals are given in Table VII. The lesions are recorded as they appeared 4 or 5 days after

¹² In order to determine whether the animals which had their lesions completely suppressed had acquired some immunity they were given a second intracutaneous injection of the same bacterium 2 weeks after the first. 4 additional normal animals were similarly injected as controls. The results may be summarized as follows: None of the animals showed any acquired immunity to either streptococcus or staphylococcus; on the contrary a sharp hypersensitivity was found in the case of streptococcus. This observation confirms the findings by Swift and his coworkers concerning the hypersensitivity induced by repeated small inocula of streptococcus in the rabbit skin (12).

the inoculations, save in the case of those of the Shope virus which were measured after 12 to 14 days.

It will be seen from Table VII that the spreading action of testicle extract considerably enhances the development of virus lesions in all dilutions, even those approximating the minimal infective dose, and in the case of all the highly various strains employed. Both Hoffman and McClean (11, 1) have made similar observations, theirs including vaccine virus and those of herpes vesicular stomatitis and foot-and-mouth disease. It would appear that the effect of dispersing filterable viruses is entirely different from that in the case of bacteria, being uninfluenced either by dilution or by the "virulence" of the strain employed. The thought suggests itself that the critical concentration of virus per unit area is the minimal infective dose, or in other words, an *infective unit of virus*.

The Effect of Spreading on Neurovaccine Virus Lesions When the Host Resistance Is Raised

Since the experiments just described were carried out on normal rabbits, we next tried to determine whether suppression by spreading took place in rabbits whose resistance had been raised by specific antiserum, as was the case with a highly virulent strain of pneumococcus.

Experiment.—8 rabbits were injected intracutaneously on one side with 5 or 6 dilutions of the standard pulp of neurovaccine virus mixed with testicle extract and the same was done on the other side, using saline solution instead of testicle extract. 0.5 cc. of each ingredient was used. Immediately afterwards 5 of the rabbits were injected intravenously with 10 cc. each of serum from rabbits recently immune to vaccinia, and another rabbit was similarly injected with 5 cc. of the immune serum. The other 2 rabbits were not injected and served as controls.

The results are expressed in Table VIII and Text-figs. 2 and 3.

An additional test was carried out with 2 rabbits in which mixtures of vaccine virus and antiserum were injected intracutaneously together with testicle extract on one side while on the other vaccine virus was introduced with antiserum alone. The 1 cc. injection was composed of 0.5 cc. of testicle extract, 0.25 cc. of vaccine virus diluted 1:100, and 0.25 cc. of antiserum. The dilutions and the results are shown in Table IX.

Table VIII and Text-figs. 2 and 3 show that there was very little if any enhancement or suppression as result of spreading the virus in

TABLE VII
Lesions Produced by Filterable Viruses Injected with Saline and Testicle Extract Respectively (Recorded at the Time of the Maximum Lesion, 5 to 12 Days after Injection)

Dilutions of standard emulsions of viruses	Levahliti neuro- vaccine virus		Noguchl testicle- vaccine virus		Cultured dermo- vaccine virus		Plain dermo- vaccine virus		Rivers' Virus III		Shope's virus (1st passage)		Shope's virus (18th passage)	
	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract	Saline	Testicle extract
1/10	5+	7+	3+	5+	4+	8+	2+	3+	±	2+	3+	8+	2+	6+
1/100	5+	8+	3+	4+	3+	6+	1+	2+	±	6+	2+	6+	2+	6+
1/500	3+	6+	3+	4+	3+	6+	1+	3+	±	2+	±	2+	±	4+
1/10 ⁴	2+	4+	3+	3+	2+	3+	±	3+	±	±	—	±	±	4+
1/10 ⁵	1+	3+	2+	4+	1+	1+	±	1+	—	1+	—	—	1+	±
1/10 ⁶	1+	2+	1+	3+	1+	1+	±	±	—	±	—	—	±	±
1/10 ⁷	±	±	1+	3+	±	±	—	±	—	—	—	—	—	—
No. of tests,.....	6		2		1		2		3		2		2	

rabbits whose resistance had been raised. That the antiserum employed was an effective neutralizing agent comparable to the anti-pneumococcus serum of a previous experiment is shown by the fact that 10 cc. of it nearly suppressed the virus infection whether it was injected with or without testicle extract.

Table IX shows that spreading enhanced the vaccinal lesions in the presence of the specific antiserum as well as without it.

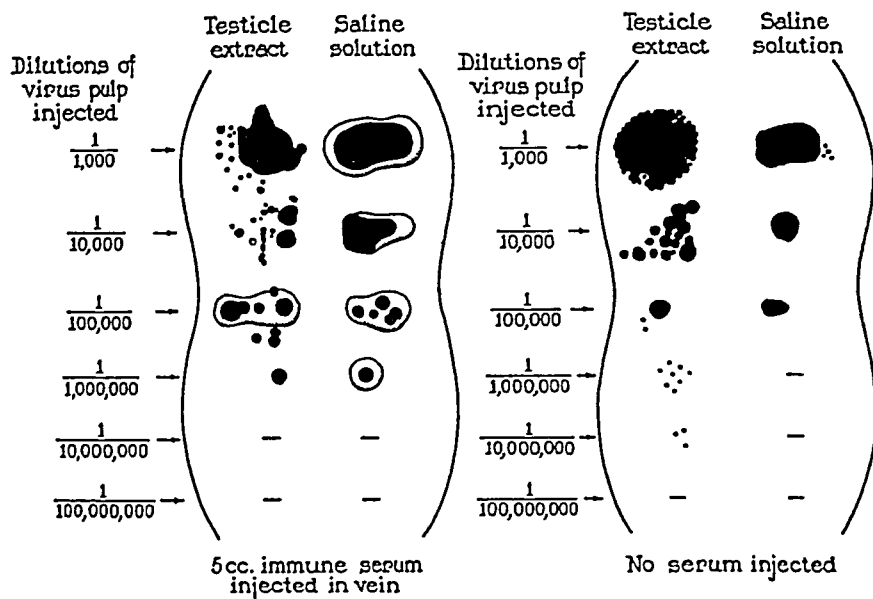
TABLE VIII

Lesions Produced by Neurovaccine Virus Injected with Testicle Extract and Isotonic Saline Respectively in Rabbits Partly Immunized with Specific Antiserum (Recorded at the Time of the Maximum Lesion, 5 to 7 Days after Injection)

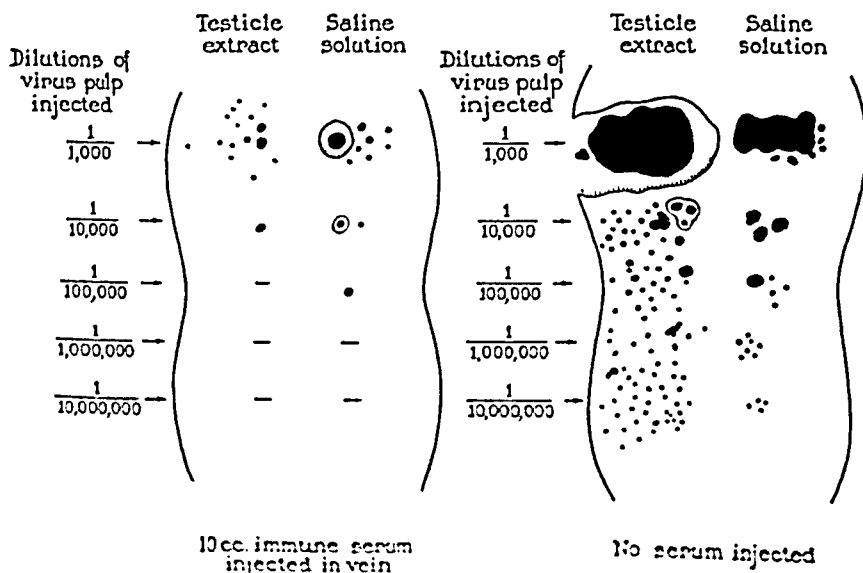
Rabbit No.	Lesions produced by progressive dilutions of vaccine virus pulp plus testicle extract						Lesions produced by progressive dilutions of vaccine virus pulp plus isotonic saline						Amount of serum injected intravenously
	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000	1/100,000,000	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000	1/100,000,000	
1	++++±	2+	2+	1+	-	-	4+	2+	2+	1+	-	-	5 Sample A
2	2+	1+	1+	-	±	-	3+	2+	±	-	-	-	10 Sample A*
3	++±	1+	-	-	-	-	2+	1+	1+	-	-	-	10 Sample B
4	1+	-	-	-	-	-	+	±	±	-	-	-	10 Sample C
5	1+	-	±	-	-	-	2+	1+	-	-	-	-	10 Sample C
6	+±	-	±	-	-	-	+	1+	-	-	-	-	10 Sample C
7	4+	3+	1+	-	±	±	2+	1+	1+	±	-	-	No serum injected
8	8+	4+	3+	3+	3+	3+	3+	2+	1+	1+	-	-	No serum injected

* Rabbit 2 was injected with 5 cc. of serum 24 hours before the skin injection, and another 5 cc. at the time when the skin was injected.

These experimental results establish the fact that the reactions to a typical filterable virus, when the infective agent is spread in the partly immune animal, differ from those when a highly virulent bacterium such as Pneumococcus Type I is spread under similar conditions.



TEXT-FIG. 2. Lesions recorded after 5 days.



TEXT-FIG. 3. Lesions recorded after 5 days.

INFECTIOUS AGENTS AND RESISTANCE TO INFECTION

Effect of Spreading on Bacteria of Moderate or High Virulence When Bacterial Extract Is Substituted for Testicle Extract

Some of the tests dealing with the effects of spreading on bacteria and viruses in the normal rabbit were repeated, using a saline extract of an invasive strain of staphylococcus as the agent for the dispersion instead of testicle extract.

Experiment.—The strain (Strain 40) was of the *aureus* type and was selected because the filtrates of its saline extracts, although spreading actively, had prac-

TABLE IX

Average of Lesions Produced in 2 Rabbits by Mixtures of Vaccine Virus and Specific Antiserum, Injected with Testicle Extract and Isotonic Saline Respectively
(Recorded at the Time of the Maximum Lesion, 5 to 7 Days after Injection)

Skin site	Vaccine virus diluted 1/100	Saline solution	Testicle extract	Dilution of antivaccinal serum (0.25 cc.)	Resulting lesion
		cc.	cc.		
	0.25 cc.	0.5	0	Undiluted	±
1	" "	0	0.5	Undiluted	±
2	" "	0.5	0	1/10	±
3	" "	0	0.5	1/10	+±
4	" "	0.5	0	1/100	3+
5	" "	0	0.5	1/100	4+
6	" "	0.5	0	1/1,000	3+
7	" "	0	0.5	1/1,000	6+
8	" "	0.5	0	1/10,000	4+
9	" "	0	0.5	1/10,000	7+
10	" "	0.5	0	0	3+
11	" "	0	0		

tically no injurious action on the rabbit skin, unlike extracts from certain other invasive strains. 0.5 cc. of extract with an equal volume of the bacterial suspensions was injected immediately after mixing, as when testicle extract was used. The results with the 8 rabbits employed are summarized in Table X.

It is evident from Table X that *Staphylococcus aureus* Strain 50 and *Staphylococcus albus* were inoculated at or below the critical concentration, and the dispersion of these bacteria by the staphylococcus spreading factor almost or quite prevented the development of lesions. In the case of *Staphylococcus aureus* Strain 40 and *B. prodigiosus* the

TABLE X
Lesions Produced by Bacteria of Low and High Virulence Injected Together with
Saline Solution and the Spreading Factor from *Staphylococcus* Respectively
(Recorded at the Time of the Maximum Lesion,
2 to 6 Days after Injection)

Dilutions of bacterial suspensions (fractions of 24 hr. agar culture)	Bacillus of Mouse Typhoid II		<i>Staphylococcus</i> <i>aureus</i> (Strain 40)		<i>B. prodigiosus</i>		<i>Staphylococcus</i> <i>aureus</i> (Strain 50)*		<i>Staphylococcus</i> <i>albus</i> *	
	Saline	Staph- ylo- coccus extract	Saline	Staph- ylo- coccus extract	Saline	Staph- ylo- coccus extract	Saline	Staph- ylo- coccus extract	Saline	Staph- ylo- coccus extract
1/200			3+	4+	3+	5+	3+	±±	3+	2+
1/1,000			1+	±±					2+	1+
1/2,000			1+	±±	3+	5+	2+	±	1+	±
1/10,000			1+	±±	3+	5+	1+	—	1+	—
1/20,000			1+	±±	3+	5+	1+	—	1+	—
1/200,000	4+	6+	1+	—	3+	1+	1+	—		
1/2,000,000	4+	6+	±	—	2+	1+	1+	—		
1/20,000,000	4+	6+	±	—	2+	1+	1+	—		
1/200,000,000	3+	4+					±	—		
1/2,000,000,000	2+	2+								
1/20,000,000,000	1+	1+								
No. of tests.....	1		3		1		2		1	

Arrows indicate minimal effective concentration of bacteria.

* Other tests have shown that more concentrated suspensions give enhanced lesions under the influence of either testicle or bacterial extract.

TABLE XI
Lesions Produced by *Neurovaccine Virus* Injected Together with Extract from an
Invasive *Staphylococcus* and with Saline Solution Respectively (Recorded
at the Time of the Maximum Lesion, 6 Days after Injection)

Dilution of virus	Rabbit 1		Rabbit 2		Rabbit 3	
	Saline	Bacterial extract	Saline	Bacterial extract	Saline	Bacterial extract
1/200	5+	7+				
1/1,000	5+	8+	3+	8+	2+	8+
1/2,000	4+	6+	4+	8+	1+	7+
1/10,000	±	3+	4+	6+	1+	7+
1/20,000	—	1+	1+	5+	±	6+
					±	4+

spreading action of the bacterial extract enhanced the lesions when the organisms were above the critical concentration, and when they were below it, suppressed them. This latter also held true for the bacillus of Mouse Typhoid II, but dilutions were not carried above the critical concentration.

In another group of experiments in which 3 rabbits were used, vaccine virus was injected in progressive dilutions in one side of the rabbit together with the staphylococcus extract, while in the other side saline instead of extract was introduced. The method was the same as when dealing with the action of testicle extract on filterable viruses. The results are expressed in Table XI.

The results shown in Table XI were similar to those where testicle extract was used for the spreading agent; namely, enhancement of the lesions at all virus dilutions.

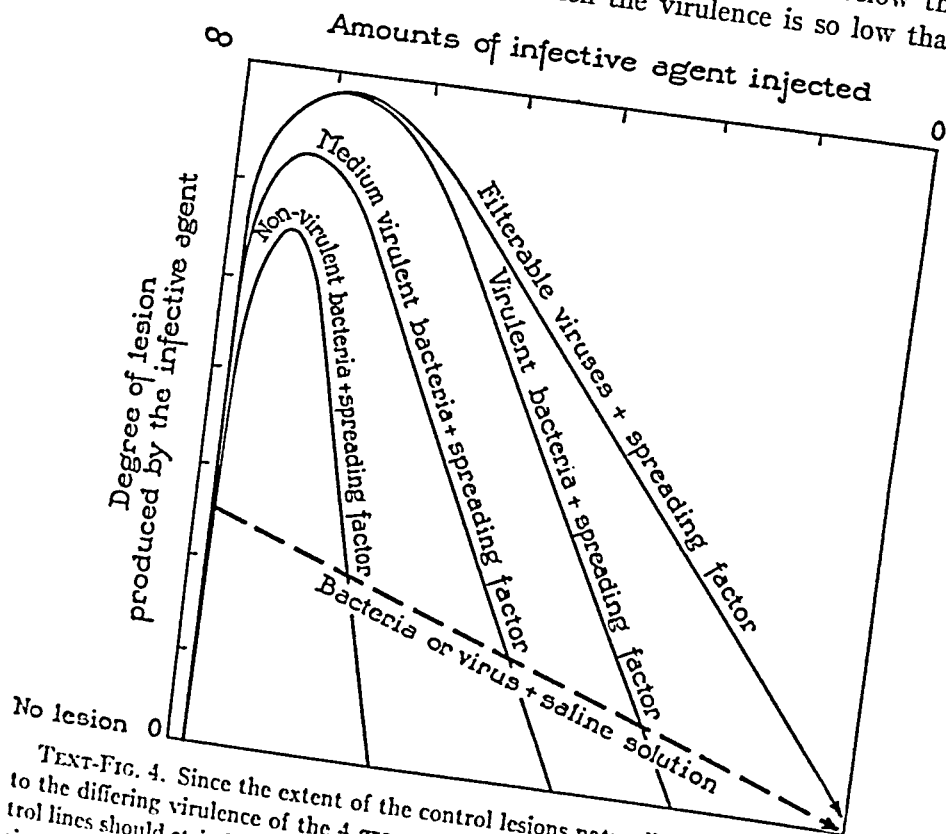
From these two sets of experiments it can be concluded that the spreading factor derived from an invasive staphylococcus has the same effect upon the fate of bacteria and viruses respectively as has testicle extract. Its effects were not limited to the class of organisms from which it derived.

DISCUSSION

If progressively decreasing doses of bacteria are injected together with extracts of testicle or invasive staphylococcus into the skin of the normal rabbit, enhancement of the lesions is produced when the number of bacteria introduced is large and suppression of the lesions when it is small. If the bacterium is highly virulent, *e.g.* Pneumococcus Type I, only enhancement of its lesions will be observed in the normal animal, whereas inhibition takes place in the partly immunized animal. On the other hand, enhancement of the lesions due to filterable viruses is observed at all the dilutions in the normal rabbit, and in the partly immunized one no inhibition or very little is observed when they are injected together with testicle or staphylococcus extracts.

From the findings it is reasonable to assume that enhancement of the bacterial lesions will be observed, first when the concentration of the infectious agent at the site of inoculation is so high that the scattering effect of dispersion fails to reduce it below a critical concentration, and second when the virulence of the inoculated bacteria is so great that local agencies are insufficient to inactivate them even

when widely separated in the lesions. Inhibition will be observed, first when the concentration of bacteria at the site of inoculation is such that the diluting effect of dispersion will reduce it below the critical concentration, and second when the virulence is so low that



TEXT-FIG. 4. Since the extent of the control lesions naturally differs according to the differing virulence of the 4 groups of infective agents, 4 thick dotted control lines should strictly speaking have been drawn in the figure. For the sake of simplicity, however, a single line common to all the controls has been introduced. The critical concentration for each of the 3 groups of bacteria appears to lie at the intersection of the straight control line with the corresponding curved line. No such intersection exists for the group of viruses.

the local protective agencies are able to inactivate the bacteria even when their number is relatively considerable. This presumptive mechanism will be easier to discuss if an example is given. Let it be supposed that a minimum of 10 bacteria, for

example, are necessary to cause lesions in a unit area of skin. If a dose of 50 bacteria is dispersed through 5 unit areas of skin a large, or in other words, an enhanced lesion will result, since each unit area gets 10 bacteria, the effective pathogenic dose; but if only 10 bacteria in all are injected, then dispersion through 5 areas of skin will result in a complete suppression of any lesion, though a small but definite one would have developed had they not been scattered.

It remains to be seen whether the behavior of other virulent bacteria spread in the normal or partly immunized host will be the same as that of those species and strains studied in this investigation. The tubercle bacilli and allied organisms constitute an interesting test material from this point of view, and they are now being investigated (13). The effect of spreading upon "attenuated" viruses would also make an interesting study.

A satisfactory explanation of the differing effects of dispersion upon bacteria and upon viruses waits upon further investigations. Testicle extract, as an agent for dispersion, would appear to be one more tool for discrimination between bacteria and viruses. The viruses studied here were not suppressed in their action when spread, no matter what the resistance of the host and the virulence of the strain. This held true even for doses closely approaching the minimal infective dose, and hence one may assume that the *critical concentration* of virus per unit area of infection is an infective unit of virus. The differences in the phenomena are presented graphically in Text-fig. 4.

Virus infections of the skin appear to be associated with local reactions different from those attending bacterial infections and it is possible that the differences may be determined, in part, by the protection afforded by the association of viruses with cells (14) and the lack of any such protection in the case of extracellular bacteria (15).

SUMMARY

Progressively decreasing quantities of bacteria of some 20 strains were utilized in experiments upon the effect of dispersing the organisms in the rabbit skin through the agency of an extract of testicle or an invasive staphylococcus. The same was done with 6 strains of filterable viruses.

The bacterial lesions were enhanced by spreading when the organ-

isms introduced were above a certain number or quantity (minimal effective concentration) and on the other hand were partially or totally suppressed when their number was less than this. Virulence and minimal effective concentration were observed to be in inverse relationship. The lesions due to the filterable viruses studied were, on the other hand, enhanced by the spreading factor even when the quantity of virus approached the minimal infective dose. This happened irrespective of whether the virus caused severe lesions or slight ones.

The highly virulent *Pneumococcus* Type I, injected into normal rabbits together with the spreading factor, yielded enhanced lesions even at practically its minimal infective dose; but when the resistance of the animal was raised with specific antiserum the lesions were totally suppressed by the experimental dispersion of the bacteria. When such an experiment was repeated on a filterable virus, vaccinia, no suppression took place as a result of the dispersion of the infective agent.

The significance of the differences in the bacterial and virus phenomena is discussed.

It is a pleasure to thank Dr. D. A. MacFadyen not only for active help in the correcting of the manuscript but also for many valuable suggestions concerning the exposition of the experimental results.

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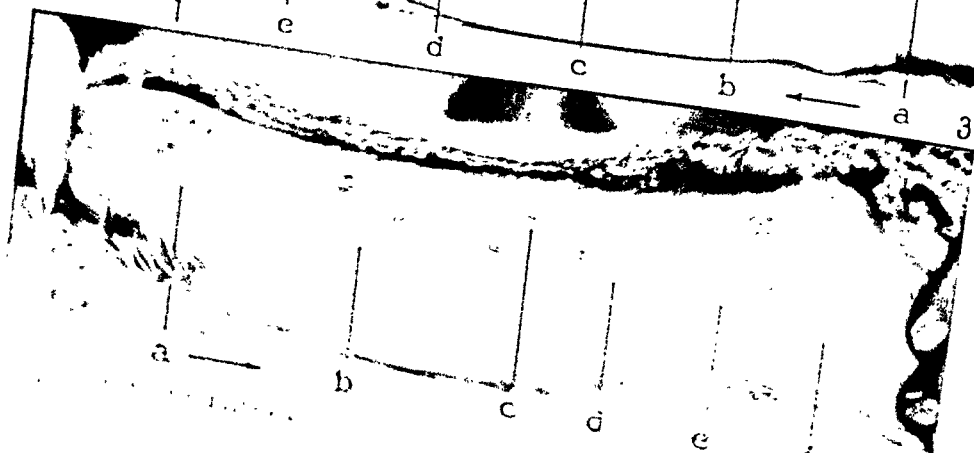
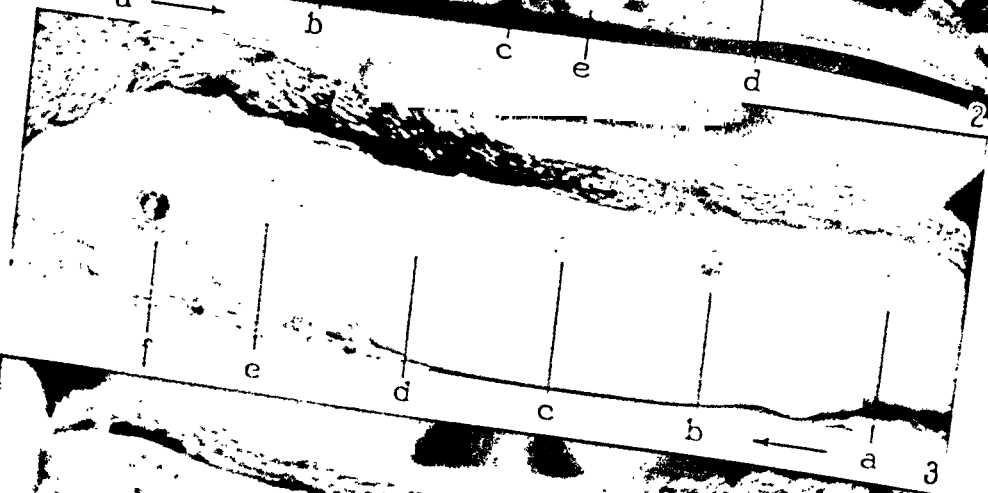
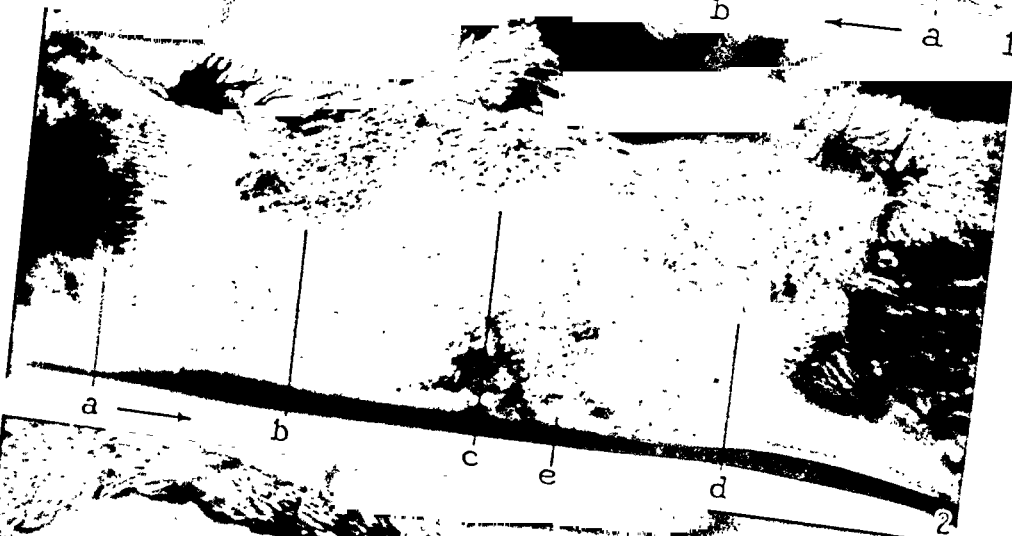
EXPLANATION OF PLATE 29

FIG. 1, *a, b, c, d, e*. Rabbit 4-16. *Right side (control)*. Lesions produced by the intracutaneous injection of increasing dilutions of a 24 hour culture of mouse typhoid bacillus, from 1/2,000,000 to 1/100,000,000, suspended in normal saline solution.

FIG. 2, *a, b, c, d, e*. Rabbit 4-16. *Left side*. Lesions produced by the same amounts of bacteria, suspended in testicle extract. Note the enhancement of all lesions and also the tendency to a pustular aspect in the lesions produced by the higher dilutions.

FIG. 3. Rabbit 4-64. *Right side (control)*. Lesions produced by intracutaneous injection of increasing dilutions of a 24 hour culture of mouse typhoid bacillus, from 1/200,000,000 to 1/20,000,000,000, suspended in saline.

FIG. 4. Rabbit 4-64. *Left side*. Results of injection of the same amounts of bacteria, suspended in testicle extract. Note the manifestly pustular character of the lesions produced by the most concentrated suspensions of bacteria (*a, b, and c*) and the complete suppression of the effects of the least concentrated suspensions (*d, e, and f*).



STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS*

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PLATE 30

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Not a few investigations have been carried out on the sensitization of animals to simple compounds known to produce hypersensitiveness in man, but the progress of this subject has been hindered to a certain extent by difficulties encountered in reproducing the experiments.

Sensitization with salvarsan has been reported by Swift (1), and with neoarsphenamine by Frei (2), and Sulzberger (3). Swift recorded general symptoms,¹ whereas in the experiments of the latter two authors the effects consisted in the development of skin lesions in the treated animals. Similarly, cutaneous hypersensitiveness was induced with phenylhydrazine by Jadassohn (4) and with *p*-phenylenediamine by Mayer (5) and Dienes (6). In the case of *p*-phenylenediamine the mechanism of sensitization can be more easily understood than in other instances because this compound, after oxidation, is apt to enter into a firm chemical union with proteins, and, in fact, *p*-phenylenediamine is used extensively as a fur dye. This interpretation is evident for the experiments of Klopstock and Selter (7) with diazonium solutions and actually their treatment produces a state of typical anaphylaxis which would seem to set this case apart from the common instances of drug and occupational allergy.

Apparently the most striking and consistent results have been obtained by Bloch and Steiner-Wourlich (8) with primulin, in animals as well as human beings, and more recently by Rackemann and Simon (9) with poison ivy in guinea pigs. The latter experiments were performed with extracts, whereas in the former a pure substance of simple composition ($C_{14}H_{11}O_4$ or $C_{14}H_{11}O_4OH$) was used which, however, has the drawback of not being easily accessible.

On attempting to reproduce, in New York, the results he had obtained in Europe with neoarsphenamine, Sulzberger (10) found that the experiments were

* The experiments have in part been briefly reported in *Proc. Soc. Exp. Biol. and Med.*, 1934, 31, 790, 1079.

¹ Other experiments along this line are critically reviewed by Frei (2).

no longer distinctly positive.² He summarizes his findings by saying that guinea pigs reacted differently when sensitizations with the same brand of neoarsphenamine were attempted in various places. In Breslau 98 per cent of the animals became sensitized, but none in New York; Zurich animals showed a state of susceptibility placing them between those of New York and Breslau in this respect. From a study with Mayer (11) of the factors involved, he concluded that the diet of the animals was of decided importance; green fodder inhibiting, dry fodder favoring sensitization.

The observations of Walthard (12) on the sensitization of guinea pigs with nickel salts³ could not be duplicated by Coca and Milford (13). Experiments by Mu (14) on neosalvarsan were definite but variable in guinea pigs, and in rabbits positive in some batches, negative in others. Frei (2) was unable to obtain clear-cut results with the latter species.

The passive transfer of human idiosyncrasy to iodoform and iodine, described by Bruck (15) and Klausner (16), has, so far, not been corroborated; and likewise Meyer (17), Bock (18), and Mayer (19) were unsuccessful in attempts to produce active anaphylaxis to *p*-phenylenediamine in guinea pigs as reported by Curschmann (20), Gerdon (21), and Mehl (22). The first two authors and Mayer (23) also reported positive transfer to guinea pigs with the serum of human beings hypersensitive to ursol, but the latter was not able to transfer hypersensitiveness from guinea pig to guinea pig.

It is apparent from a survey of the work bearing on the subject that the conditions which influence the experiments are not yet fully understood and so far, in most cases, the lesions obtained in animals have not been equal in intensity to those occurring in human beings.

Other pertinent questions also are still unsolved. It is not known whether the capacity to induce sensitivity is connected with certain peculiarities in chemical constitution, and with a number of substances to which some human individuals are hypersensitive it has not been possible, as yet, to induce such a state in animals. Moreover, the mechanism underlying the sensitization effects is uncertain, as is their relationship to the condition of anaphylaxis as produced by typical antigens.

² We also had irregular results with neoarsphenamine, but the results were markedly improved by using arsphenamine without neutralization. According to a personal communication by Sulzberger, he encountered difficulty in repeating the experiments by Jadassohn on phenylhydrazine, also, whereas in a small series of animals we found definite evidence of sensitization.

³ An experiment of our own with intracutaneous injections of small amounts in a few animals was negative.

For the reasons stated it seemed desirable to search further for sensitization effects which could be obtained regularly with easily available simple substances of known chemical composition.

EXPERIMENTAL

Technique—Guinea pigs were used as experimental animals and for the sensitization various procedures proved effective. In a large part of the experiments the method of repeated injection of small quantities was adopted, but other methods were used besides; *i.e.*, single injections, or applications of an olive oil solution on the skin, or salve. A more extensive study of various methods of administering the substances will be necessary finally to evaluate their comparative merits.

Injections were made, after clipping the hair with an electric hair clipper, with a No. 26 short bevel needle into the skin of the back, near to the surface, without abrading the skin. The volume of the injected fluid was always 0.1 cc. Olive oil solutions (1 per cent) were administered by gentle spreading with a glass rod. White guinea pigs 350–450 gm. in weight were used throughout.

For treatment and tests the substances were weighed, dissolved in a small amount of 1 per cent saline, or alcohol if they were too insoluble in water, and made up to the required dilution with saline. Usually a stock 0.3 per cent solution in alcohol was kept and the required saline dilutions made up before injections. In the case of relatively unstable substances, as neosalvarsan, *p*-aminophenol, and *p*-phenylenediamine, the dry substance was dissolved in saline before injection.

The substances used were commercial preparations, recrystallized when necessary, excepting several substituted (Cl, NO₂) benzenes (prepared in this laboratory, or obtained from the chemical laboratory of the University of Amsterdam⁴), *m*-chloroacetylaminophenol and chloroacetyl-*p*-chloroaniline,⁵ dextran,⁶ and carbohydrate preparations (made by the authors).

Reactions were read on the day following the test injections, which were made on the flanks of the guinea pigs. The hair was clipped with an electric clipper and the tests read mostly after application of a depilatory. Intracutaneous injections were made as described above.

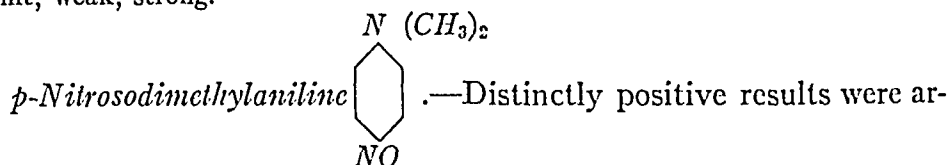
In the tables figures give diameters of the lesions in millimeters, the shade of which is described as colorless (c.), almost colorless (a.c.), faintly pink (f.p.), pale pink (p.p.), and pink (p.). Other designations are negative (neg.), almost negative (a.neg.), flat (fl.), faintly elevated (f.el.), slightly elevated (sl.el.), elevated (el.), and markedly elevated (m.el.). Insignificant reactions, namely small elevations at the place of puncture, frequently present in non-sensitized animals,

⁴ To Professors Wihaut and Holleman we are greatly indebted for furnishing us with these preparations.

⁵ Available through the kindness of Dr. Walter Jacobs.

⁶ Kindly furnished by Dr. Hibbert of Montreal.

are designated as nodules (nod.). Livid center (liv.c.) or necrotic center (necr.c.) is used to describe stages of necrosis. Oil contact tests are rated as negative, faint, weak, strong.



rived at with *p*-nitrosodimethylaniline. The use of repeated injections of small quantities was adopted following the recommendation of Kolle (24), who, in this way, in experiments on salvarsan induced a condition in guinea pigs in which they succumbed after administration of quantities otherwise well tolerated. The fact that nitroso-

TABLE I

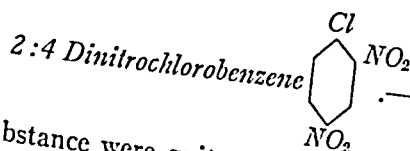
Animals Tested by Intracutaneous Injection of 1/30 Mg. Nitrosodimethylaniline

Treated		Controls	
Guinea pig No.	Lesions	Guinea pig No.	Lesions
1	9 x 7, p., sl.el.	7	c., nod.
2	11 x 9, p.p., fl.	8	4, p.p., nod.
3	9 x 6, p., sl.el.	9	c., nod.
4	14 x 13, p.p., m. el.	10	f.p., nod.
5	9, p., el.	11	neg.
6	9, p., el.	12	neg.

dimethylaniline is capable of producing hypersensitiveness in human beings was brought to our knowledge by van der Scheer, who had observed a striking instance of such an affection. A batch of animals were given repeated intracutaneous injections of 1/30 mg. each in 0.1 cc. saline solution on the back, two a week for 10 weeks, and tested on the side with the same dose 3 weeks after the last treatment (Table I). Beginning with the 2nd week the injections were followed in all the animals by reactions, which gradually became stronger; not seldom a decrease was noticed later in the course. The lesions, which were pinkish to pink and elevated, were fully developed after 1 day. In normal control guinea pigs but slight effects were produced by injections of the substance into the skin.

A second experiment showed that much smaller doses may cause

sensitization. Thus it was seen that eighteen daily injections of 1/500 mg. produced unquestionably positive effects, and in another experiment, which would require repetition, even doses of 1/5000 mg. gave noticeable results. Sensitization effects were also brought about by rubbing guinea pigs with vaseline containing 5 per cent of the compound.



—The effects obtained with this substance were quite similar to those just described (Figs. 1 and 2) but in general still stronger. The fact that sensitization occurs in

TABLE II
Guinea Pigs Tested by Intracutaneous Injection of 1/100 Mg. of 2:4 Dinitrochlorobenzene

Guinea pig No.	Treated	Guinea pig No.	Controls
	Lesions		Lesions
13	7 x 5, p.p., cl.	18	6, p.p., sl.cl.
14	12, p.p., m.cl., liv.c. 4½	19	4, f.p., f.cl.
15	16 x 15, p., m.cl., liv.c. 3	20	3, a.c., nod.
16	9, p.p., cl.	21	p.p., nod.
17	17 x 15, p.p., cl., liv.c. 3	22	5, f.p., sl.cl.

human beings was called to our attention by Professor Zangger of Zurich. Reports on cases of human hypersensitiveness have been published by Wedroff (25, 26), who observed greatly increased hypersensitivity to the compound in many of the exposed factory workers.

One lot of guinea pigs, tested intracutaneously with 1/100 mg. dinitrochlorobenzene 2 weeks after a course of ten daily injections of 1/400 mg. on the back, showed strong reactions, three out of five with livid centers (indicative of necrosis) (Table II). Distinct reactions had been obtained in these animals a week earlier, following injections of 1/400 mg. A further experiment gave striking results when the animals were tested with 1/400 mg. intracutaneously 1 week after a course of two weekly injections of the same quantity over a period

of 10 weeks (Table III). The lesions, in four of the six animals, showed necrotic centers.

Again, as with nitrosodimethylaniline, reactions developed during the course of injections after the 1st week, later reaching a maximum.

In over twenty batches of guinea pigs treated with the substance at different times practically all animals responded with definitely increased hypersensitivity, although the effects were not equally good and not always so strong as those tabulated, and there were marked individual variations within each batch as is evident from a glance at the tables.

TABLE III

Guinea Pigs Tested by Intracutaneous Injection of 1/400 Mg. of 2:4 Dinitrochlorobenzene, and Application of a 1 Per Cent Solution in Olive Oil

Treated				Controls			
Guinea pig No.	Intracutaneous injection			Oil solution	Guinea pig No.	Intracutaneous injection	Oil solution
23	12,	p.p., sw., necr.c. 5		Weak	29	a. neg.	Neg.
24	9,	p., m.el.		Strong	30	4, f.p., sl.el.	Neg.
25	12,	p.p., m.el., necr.c. 4		Strong	31	4, f.p., sl.el.	Neg.
26	17,	p.p., el., necr.c.		Strong	32	a. neg.	Neg.
27	11,	p., el., necr. c.		Strong	33	a. neg.	Faint
28	11 x 7,	p., el.		Strong			

Apparently, a still better way than intracutaneous injection to demonstrate the increased sensitivity of treated animals was to spread a drop of a 1 per cent solution of dinitrochlorobenzene in olive oil on the skin with a glass rod. In sensitive animals the areas in contact with the substance showed a faint pinkish color after a few hours which increased to a distinct pink or red on the following day, and were, in many cases, elevated or actually swollen, in striking contrast to the negative controls (Table III). The same method proved to be applicable in other cases; *e.g.*, with nitrosodimethylaniline.

Also, by treating the skin with oil solutions of dinitrochlorobenzene, it was possible to sensitize guinea pigs.

In other tests alcoholic solutions were used as done by Wedroff (26), who described reactions after the application of alcoholic solutions of

dinitrochlorobenzene in sensitive patients. With this method well sensitized guinea pigs showed reactions following the application of 1 drop of a 1:100 or 1:1000 solution of dinitrochlorobenzene which compared to the degree of sensitivity of a large part of Wedroff's patients although a number of them reacted to dilutions much higher still.

In the preceding experiments repeated injections were administered, but it was found that positive results can be obtained with but few applications. Even after the first injection there was in a batch of four animals a noticeable effect when they were tested intracutaneously a week later, and with the rubbing test after 1 more week, two strong, one marked, and one faint reaction were obtained, whereas the controls were practically negative. In a few other experiments designed to vary the method of sensitization it was found that following a single injection the effect seemed to be somewhat but not much more pronounced when tests were made after an interval of 4 weeks, than after 1. A considerably larger dose than that commonly used, namely five simultaneous intracutaneous injections of 1/100 mg., was not superior to one injection of 1/400 mg. when the animals were tested after a rest period of 4 weeks. That a course of several injections over a period of weeks gives better results than a single injection was indicated by the following experiment. Of two batches of animals given two injections of 1/400 mg. 3 days apart, one in which the injections were continued once a week for 5 weeks showed much stronger reactions than the other batch, on testing 1 week after the last injection given to the second lot.

In preliminary experiments subcutaneous or intravenous, instead of intracutaneous injections, gave only slight sensitization effects, and repeated instillations of a dilute saline solution into the eye were ineffective.

Chloro and Nitro Substituted Benzenes.—Definitely positive sensitization effects were observed with a number of chloro and nitro substitution products of benzene: 1:2:4 trinitrobenzene, picryl chloride, and four dichlorodinitrobenzenes (1:3:4:6, 1:3:2:5, 1:2:4:5, 1:4:2:6), while three dichloronitrobenzenes (3:5, 3:4, 2:5), *p*-chloronitrobenzene, *m*-dinitrobenzene, 1:3:5 trinitrobenzene, picric acid, and four

compounds containing only chlorine as substituents, namely *p*-dichlorobenzene, 1:2:4 trichlorobenzene, 1:2:4:5 tetrachlorobenzene, and hexachlorobenzene,⁷ failed to yield evidence of sensitization.

Of substituted benzenes of the type R:NO₂:NO₂, 1:2:4, tested for sensitization, 2:4 dinitrofluorobenzene, 2:4 dinitrobromobenzene, 2:4 dinitroiodobenzene (and 1:2:4 trinitrobenzene) were distinctly active, and 2:4 dinitrophenol gave a slightly positive result which would require confirmation. With 2:4 dinitroaniline and 2:4 dinitrophenylmercaptan⁸ negative results were obtained.

Among other substances marked results were obtained with *m*-chloroacetylaminophenol and definitely positive effects with chloroacetyl-*p*-chloroaniline, *p*-aminophenol, and *p*-nitrosophenol.

With some substances, excitants of human idiosyncrasies, namely quinine, resorcinol, and acetylsalicylic acid, our methods failed to give results. Likewise negative were experiments with substances chosen because of their highly irritating properties, to wit turpentine, croton oil, acridine, as well as the carcinogenic compound dibenzanthracene. Again, a number of dyes, among them azodyes obtained by coupling resorcinol with diazo compounds, were ineffective, or not definitely active. An exception to this seemed to be resorcinoldisazo-*p*-suberanilic acid.⁹ Of six animals given repeated injections of 1/50 mg. of this dye for 10 weeks one showed unmistakable and one somewhat increased sensitivity on reinjection after a month of rest. Finally it is worth noting that the method of injecting minute quantities intracutaneously did not induce hypersensitivity of the skin in some preliminary experiments, using carbohydrates of *V. cholerae* and *B. pseudoanthracis* and dextran.¹⁰ Of course, the negative results reported cannot be taken as final, since other methods may still prove effective.

Tests for Specificity.—The specificity of hypersensitiveness has been

⁷ The solutions used were stabilized by the addition of some guinea pig serum.

⁸ Dissolved with the aid of dilute ammonium hydroxide.

⁹ Previously shown to be able to induce specific anaphylactic shock in guinea pigs sensitized with an azoprotein made from suberanilic acid (Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1933, 57, 633.

¹⁰ Zozaya, J., *J. Exp. Med.*, 1932, 55, 325.

studied by several authors in human beings,¹¹ but, so far, not many observations have been reported in animals (Mayer (19)). We have performed such experiments, first with guinea pigs selected for their strongly developed sensitivity to *p*-nitrosodimethylaniline or 2:4 dinitrochlorobenzene, between which sharp specificity was noticed, the animals reacting to the substance with which they had been treated and not significantly with the other, as is shown in Table IV.

TABLE IV
Guinea Pigs Tested by Intracutaneous Injection of 1/50 Mg. *p*-Nitrosodimethylaniline or 1/400 Mg. 2:4 Dinitrochlorobenzene

Sensitive to	Guinea pig No.	Tested with	
		Nitrosodimethylaniline	Dinitrochlorobenzene
Nitroso-dimethyl-aniline	34	9 x 7, p.p., f.el.	a. neg.
	35	12 x 9, f.p., sl.el.	3, f.p., f.el.
	36	12 x 7, p.p., cl.	3, f.p., nod.
	37	11 x 10, p.p., cl.	c., nod.
	38	11, p., cl.	5 x 3, p.p., sl.el.
	39	11, p., cl.	4 x 3, p.p., sl.el.
Dinitrochlorobenzene	40		9, f.p., cl., liv.c.
	41	c., nod.	5, f.p., sl.el., liv.c. 3
	42	neg.	10, p.p., cl., liv.c. 3
	43	a. neg.	10 x 8, p.p., cl.
	44	a. neg.	9 x 8, p.p., cl.
	45	c., nod.	6, p.p., sl.el.
		3, f.p., sl.el.	

Guinea pigs hypersensitive to *p*-nitrosodimethylaniline did not exhibit increased reactions to some structurally related compounds as *p*-nitrosophenol, dimethylaniline, and nitrosobenzene. One guinea pig sensitive to *p*-nitrosophenol showed a distinct reaction when tested with nitrosodimethylaniline.

In the series of substituted benzenes of the type 1:2:4 = R:NO₂: NO₂ the nitro, fluoro, chloro, bromo, and iodo compounds sensitized, as mentioned previously, and gave cross-reactions among themselves; the corresponding amino and sulphydryl compounds (and *m*-dinitrobenzene)

¹¹ For bibliography see Landsteiner K., Die Spezifität der serologischen Reaktionen, Berlin, Julius Springer, 1933, 95.

which did not sensitize gave no, or much weaker, reactions in animals sensitized with other substances of the series. The action of 2:4 dinitrophenol was equivocal both as a sensitizing agent and in the tests. Furthermore, solutions of 2:4 dinitrobenzoic acid, 2:4 dinitroanisole, 2:4 dinitrotoluene, and 2:4 dinitrodiethylaniline did not react with pigs hypersensitive to 2:4 dinitrochlorobenzene. With these substances no sensitization experiments were made. Additional tests on animals sensitized to 2:4 dinitrochlorobenzene were made with numerous chloro and nitro substituted benzenes. Definite cross-reactions were observed in several cases, namely with picryl chloride, and two dichlorodinitrobenzenes, 1:2:4:5 and 4:6:1:3. These substances belong among those found to produce sensitization. Specificity tests were performed with the following compounds, also: picryl chloride (A), 1:4:2:6 dichlorodinitrobenzene (B), 1:3:2:5 dichlorodinitrobenzene (C), 4:6:1:3 dichlorodinitrobenzene (D) and 1:2:4:5 dichlorodinitrobenzene (E), on a limited number of animals sensitized to these substances. The specific character of the reactions was evidenced by the fact that in each batch of animals the reaction with the homologous compound was stronger, or not weaker than those with other substances. Definite cross-reactions were observed in the following cases: A with B, B with A, C with A and B, D with A, and E with A and D, where the first letter refers to the substance used for sensitization, and the others to the compounds used for the skin test.

Selected animals sensitized by repeated injections with neoarsphenamine, nitrosodimethylaniline, *p*-phenylenediamine, and resorcinol-disazo-*p*-suberanilic acid respectively, were each injected with all four of the substances. The strongest reaction, in each case, was given by the homologous substance, but the guinea pigs hypersensitive to *p*-phenylenediamine showed marked reactions with *p*-nitrosodimethylaniline, as well. This last reaction was regular and quite definite.

DISCUSSION

The essential outcome of the foregoing studies is that sensitization effects have been obtained with simple compounds, which are easily reproducible and for that reason seem to offer an advantage for further studies. From the experiments it would appear that there are a large number of substances of different composition by means of which a

state of hypersensitiveness can be induced in animals. Yet substances gave negative results with the methods used although they were structurally quite similar to those yielding positive effects.

On considering the properties which may characterize compounds causing increased sensitivity it would seem possible that the ability to irritate the tissues plays a rôle, since most of the substances that were found to produce sensitization are irritating. Primulin used by Bloch and Steiner-Wourlich (8) in human beings and animals, and extracts of *Rhus toxicodendron* (Rackemann and Simon (9)) were likewise found to produce local lesions. Still there are examples to the contrary, namely *p*-phenylenediamine which has little effect upon the skin of normal animals and yet readily induces the sensitization phenomenon, and other compounds that are highly irritating and do not do so.

As to the mechanism of hypersensitiveness to simple compounds, it is the simplest explanation and in line with the opinion of most authors to relate them to the familiar processes of immunization, especially in view of the specificity of the reactions. The chief difficulty in the way of adopting this view is the uncertainty still prevailing about the possibility of demonstrating circulating antibodies even in pronounced cases of human drug idiosyncrasy (see Coca (27)). In the literature there are a number of reports on successful Prausnitz-Küstner reactions in such cases, but they have been questioned by others and are not generally accepted.¹² More recent reports on passive transfers were those communicated by Frumess (28) on 2:4 dinitrophenol and by Ensbruner (29) on neosalvarsan, which, if confirmed, would be of great significance.¹³

The fact that all parts of the skin become sensitive following injection at one site would indicate either that the substance itself is transported, or that some antibody-like substance (or, possibly, a product derived from the inciting compound) manufactured at the site of injection, is responsible for the spread of sensitivity. The first

¹² Birnbaum, O., *Centr. Haut- u. Geschlechtskrankh.*, 1934, 49, 97.

¹³ The question of to what extent circulating antibodies are the necessary concomitant of immunological responses, is discussed by Zinsler, H., in Jordan, E. O., and Falk, I. S., *The newer knowledge of bacteriology and immunology*, Chicago, University of Chicago Press, 1928, 721.

hypothesis is rendered improbable, if, as Rackemann and Simon found, and as seems to be suggested by preliminary experiments of our own, the mode of introducing the substance, namely cutaneous application, is of significance for the success of the experiment. Another conceivable explanation for increased reactivity, namely accumulation of the substance injected into the body, appears to be excluded on quantitative grounds, since the sensitization effects are of long duration and can be obtained with amounts which altogether would not suffice to produce local lesions.

As pointed out in the review of the literature, immunization by simple substances could be explained if one might assume a combination of the compounds with protein, which is probable in the case of *p*-phenylenediamine.¹⁴ In this connection it should be mentioned that recently Horsfall (30) reported on the sensitization of rabbits to formaldehyde by immunizing them with formalinized proteins. Such a mechanism would appear to be more probable than the supposition that the substances act as antigens by themselves since even with bacterial carbohydrates, which as a class can be supposed to have some antigenic activity, skin sensitization in guinea pigs has, so far, not been attained. With the substances dealt with in the present paper and others known to be the cause of idiosyncrasies, the assumption of the formation of antigenic conjugates in the animal is not so obvious. However it is known that in 2:4 dinitrochlorobenzene, indeed in most chloro and nitro substituted compounds mentioned in this paper, a chlorine atom or a nitro group is but loosely bound (31), so that some of these substances combine with bases, much like acyl chlorides. With the other compounds, for instance nitrosodimethylaniline, one must bear in mind that changes may occur in the animal body by which they acquire the capacity to enter into antigenic combinations with other substances;¹⁴ and the possibility that the mechanism of sensitization may be different with various classes of compounds.

¹⁴ For a thorough discussion and study of the chemical processes involved in dyeing with *p*-phenylenediamine and the chemical changes of aromatic bases in the animal body we refer to the studies of Cox, H. E., *Analyst*, 1929, 54, 694; 1933, 58, 738; 1934, 59, 3; and Kracke, R. R., and Parker, F. P., *J. Lab. and Clin. Med.*, 1934, 19, 799.

An investigation of these questions is under way particularly with regard to the fact that some of the substituted benzenes with easily detachable substituents did not elicit sensitization.

A closer study of a variety of properly selected compounds, as well as a search for antibodies and desensitization effects may serve to throw light upon the pending questions.

SUMMARY

Experiments on the sensitization of guinea pigs with simple chemical compounds are described. Positive effects were obtained by the administration of small quantities, namely fractions of milligrams, with 1:2:4 chlorodinitrobenzene, *p*-nitrosodimethylaniline, 1:2:4 trinitrobenzene, picryl chloride, four dichlorodinitrobenzenes, and a number of other aromatic compounds. Several substances chemically similar to those enumerated gave negative results. The first named compound is known to produce hypersensitiveness in human beings, a large number of cases having been observed in factory workers.

The mechanism of these effects is discussed.

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EXPLANATION OF PLATE 30

FIG. 1. Animal sensitized to 2:4 dinitrochlorobenzene and injected intracutaneously with 1/400 mg. of the substance.

FIG. 2. Untreated animal injected in the same way.



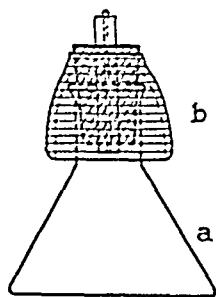
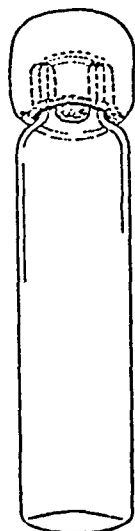
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FIG. 1. Animal sensitized to 2:4 dinitrochlorobenzene and injected intracutaneously with 1/400 mg. of the substance.

FIG. 2. Untreated animal injected in the same way.

tant deterioration of the enzyme took place in several weeks. Standardization tests of its rate of digestion at 37°C. were carried out with specimen cultures. A solution strong enough to digest the clot in 45 to 60 minutes proved best, as yielding numerous living cells in not too long a time, stronger solutions tending to kill them. Digestion was started with 4 cc. of trypsin solution to each culture, poured on after removal of the supernatant fluid; and 30 to 60 minutes later this was replaced with 2 cc. more, and sometimes replaced yet again in case the skim of clot immediately about the liberated tissue fragments had not wholly digested away. A magnifying glass was required to make certain of this. All the digests as poured off were added to the supernatant culture fluid, the serum component of the latter acting to check further digestion; and a final dilution was done to 160

TEXT-FIG. 1. $\times \frac{1}{2}$.TEXT-FIG. 2. $\times \frac{1}{2}$.

cc. with gelatin Tyrode. Except when the liberated cells were to be submitted to ultraviolet light the Tyrode employed with them regularly contained $\frac{1}{2}$ per cent gelatin, since this acts to prevent mechanical injury to cells repeatedly pipetted while in suspension (4). The abbreviations p. Ty. and gel. Ty. will be used in the protocols for plain and gelatin Tyrode respectively.

The diluted digest was cloudy with individual cells. Forcible pipetting of the tissue fragments to set more of them free liberated fibrils as well and was avoided. The undigested debris was removed as follows:—

The cell suspension was passed through two layers of very fine gauze tied like a bag into a thistle tube, and then through two of washed, close-textured lens paper. Slow centrifugation was done for 5 minutes in four 50 cc. pyrex tubes closed with corks having a central core to keep them in place and a flange to protect the lip

furthermore the growths induced by the virus regularly retrogress and disappear, and they have sometimes a large inflammatory element.

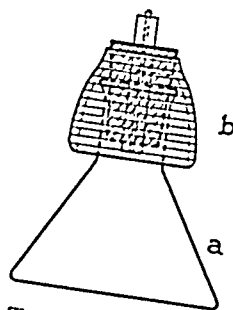
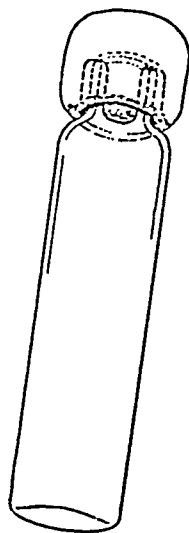
The Cell Material

When a culture containing tissue that has grown out into a plasma clot is submitted to a trypsin solution (3) many of the proliferating cells "let go hands" as the clot digests and round up separately like leukocytes. They can now be washed and plated in plasma, there to grow anew. For present purposes they have been exposed instead to virus-containing fluids, treated in various ways, and inoculated into animals susceptible to the viruses.

Rabbit embryos 18 to 24 days old were procured aseptically, decapitated, and hashed in a grinder. The fragments were washed three times by allowing them to settle out of suspension in Tyrode's solution containing $\frac{1}{8}$ per cent gelatin, and were then implanted in a 1:3 mixture of rabbit plasma and Tyrode solution. To every 25 cc. of plasma, 1.8 cc. of 1 in 1000 heparin solution was added at the time of collection. Pyrex flasks of special shape served as culture chambers. They were Ehrlenmeyer in type, of about 35 cc. capacity, with an unusually broad and flat bottom (Text-fig. 1 *a*); and they were closed with flanged stoppers (*b*),—made by cutting off both ends of an ovoid 20 cc. rubber bulb and slipping it over a rubber stopper,—thus protecting the lip of the flask from bacterial contaminants which might "creep" into the medium. A thick-walled, cotton-plugged, capillary tube, extending through the stopper, provided for readjustments of air pressure. $1\frac{1}{2}$ cc. of the suspension of the tissue fragments was put into each flask, followed by $\frac{1}{2}$ cc. of rabbit plasma, with brief agitation; and after a few minutes, when clotting was complete, 2 cc. of a 1:3 mixture of rabbit serum and Tyrode was superimposed. From eight to ten flasks were used for each sowing,—which was done in a room ventilated with filtered air and sprayed beforehand to carry down dust. The cultures were washed every 2–3 days with plain Tyrode, and the supernatant fluid renewed. Profuse growth by the end of the period was the rule. Contamination was infrequent. Just prior to the digestion to free the cells, after 2 to 7 days of incubation, the cultures were searched with a microscope, and any showing bacterial colonies were discarded.

The trypsin solution was prepared by dialyzing Fairchild's trypsin against several changes of distilled water at 4°C. until the enzyme precipitated out. The supernatant was discarded, the sediment made to 0.9 per cent NaCl by adding concentrated salt solution, further diluted to 5 per cent in terms of the original material, and passed through a Berkefeld filter. With phenol red the reaction of the fluid proved to be about pH 6.5. It was stored at -5° to -8° C. in pyrex tubes capped with rubber corks; and just prior to use a tube was thawed and diluted with Tyrode to $\frac{1}{2}$ per cent or 1 per cent, depending on its activity. No impor-

tant deterioration of the enzyme took place in several weeks. Standardization tests of its rate of digestion at 37°C. were carried out with specimen cultures. A solution strong enough to digest the clot in 45 to 60 minutes proved best, as yielding numerous living cells in not too long a time, stronger solutions tending to kill them. Digestion was started with 4 cc. of trypsin solution to each culture, poured on after removal of the supernatant fluid; and 30 to 60 minutes later this was replaced with 2 cc. more, and sometimes replaced yet again in case the skim of clot immediately about the liberated tissue fragments had not wholly digested away. A magnifying glass was required to make certain of this. All the digests as poured off were added to the supernatant culture fluid, the serum component of the latter acting to check further digestion; and a final dilution was done to 160

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(Text-fig. 2). The bottoms of the tubes had been flattened, so that the cells might come down in a thin layer; for they proved difficult to resuspend without damage when gathered into a mass. Flat discs of rubber and wood at the bottom of the centrifuge shells supported the tubes. The cloudy supernatant was drawn off through a capillary with a recurved end to prevent sucking up of the sediment, which was resuspended in 45 cc. of gel. Ty. for each tube. A drop of the material now showed great numbers of rounded, individual cells and occasional small, loose, grape-like clusters. Repeated filtrations and pipettings, with the end of the 50 cc. pipette held against the tube bottom, sufficed to remove or break up the latter. Centrifugation was now done as before, for 10 minutes, the supernatant discarded, the cells made into a suspension of slightly greater bulk than was needed for the experiment; and they were passed again through doubled lens paper, examined microscopically, and repipetted and refiltered if necessary. The ultimate, somewhat cloudy suspension showed the cells as separate spheres (Fig. 1) with, rarely, two or three joined by flat surfaces. Preparations stained with Loeffler's methylene blue were searched for bacteria. They were not always absent, since the liberation by trypsin of a single, overlooked colony in one of the many flasks of each digestion sufficed to contaminate the material. But they were found in only three of twelve instances and then in small number. All three instances happened to be those of experiments selected for detailed report here because of their comprehensiveness. In each case the contaminating organism proved to be non-pathogenic, as will appear when the protocols are given.

For some test mixtures the cells were killed by heating a part of the final suspension in a water bath at 53°C. for 15 minutes, or by exposing it to ultraviolet light. When light was used the preliminary washings and suspensions were carried out with p. Ty., the material was put in a flat-bottomed dish, covered with a sheet of quartz glass, and exposed to a mercury vapor, quartz lamp placed vertically above and 48-50 cm. away. A thick sheet of aluminum under the dish effectually conducted heat away during the exposure, the thermometer showing a rise of only a fraction of a degree C. at most. The cells killed by heat withstood the subsequent washings well, whereas those rayed for 10 minutes became so fragile that some break-up and loss occurred during even the most gentle pipetting. This loss did not alter the experimental results significantly as the protocols attest. Cells rayed for only 5 minutes stained like dead cells with neutral red, and had but a slightly increased fragility.

Several drops of the ultimate inocula, cells which had been exposed to a virus, were examined microscopically just prior to injection, on a slide coated with neutral red and ringed with vaseline. No secondary clumping of the cells had occurred. A large proportion of those not heated or rayed segregated the dye into vacuoles, showing that they were alive; and the appearance of others indicated that they were living but of different sort. Dead cells did not segregate the dye or show protoplasmic movement, and their nuclei were sharply outlined. Rayed ones were often fragmented or fatty.

The Viruses and the Immune Sera

Dr. Rivers kindly provided us with vaccine virus of the New York City Board of Health strain, in the form of a 28th subculture with chick embryo tissue and Tyrode *in vitro*. The pathogenicity of the strain had been revived twice by intratesticular inoculation into susceptible rabbits (5). For our own purposes it was propagated further in the testicles of rabbits.

The lesions caused by intradermal injection of the culture strain of virus have been studied by Rivers and Ward (6). When the liberated cells of our experiments were exposed to it briefly and washed and injected intradermally in rabbits at which the lesions appeared and spread, as well as their character, proved of importance. When but little virus was carried by the cells, or it was attenuated, the lesions were small and nodular, each nodule consisting of a necrotic focus with profuse cellular proliferation round about, as the microscope showed. Such lesions appeared relatively late, slowly increasing in size at a time when those due to more active material were already retrogressing in the same animal. Active virus caused discoid, cutaneous thickenings that were often several centimeters across, with more or less extensive vesiculation and superficial necrosis, in some instances hemorrhagic. Generalized pocking was rarely encountered, even when a number of large local lesions had been induced.

We are indebted to Dr. Shope for three strains of the virus causing the fibroma of rabbits. It was received as glycerinated tissue. The first strain tested gave rise to discoid, raised, red thickenings when a Tyrode extract of the ground tissue, cleared with the centrifuge, was inoculated into the skin; but the thickenings consisted in the main of a brawny edema with secondary pressure necrosis, pronounced reactive inflammation round about, and, exceptionally, small foci of highly abnormal, large cells of fibroblastic type, showing occasional mitotic figures. This strain (Strain A of Shope) did not fulfil our need for a virus inducing frank cell proliferation. However the others (B and C) yielded fibromatous growths like those pictured by Shope. They were used in the work. The rapidly growing, ruddy, sharply defined, spherical or discoid skin nodules frequently became capped with a broad vesicle, and later underwent a central pressure necrosis and ulceration (Fig. 2). When attenuated the virus yielded merely discoid thickenings or red indurations. Andrewes has already noted the differences in the virus strains (7).

Variations in individual susceptibility of the inoculated rabbits were pronounced, and they proved to be now a help now a hindrance in the work, bringing out or obscuring differences in the pathogenicity of the inocula. The Shope lesions were much more markedly conditioned by the body state than those of vaccinia. Thin or sick individuals tended to yield small growths or none at all; and a change for the better soon after inoculation was not infrequently attended by the appearance of the fibroma at a time when the large growths in vigorous, susceptible animals receiving the same material were already retrogressing.

The virus suspensions were mostly procured by grinding the infected tissue in a mortar with gel. Ty. and a little sterile sand. Glycerinated or fresh vaccinia testicle was used, and subcutaneous fibroma procured on the 7th to 10th day of growth. A special technique was developed to remove cells and particulate matter from the extracts. The fluid was first spun slowly, and the supernatant was transferred to 15 cc. tubes and centrifuged at high speed for 20-25 minutes with renewed transfer and rapid spinning once again. After each centrifugation a red-hot monel metal disc fixed on a wire was held above the meniscus until the surface layer began to bubble. Cells that had risen to the surface with air or fatty material were thus killed. Then the clear fluid from the middle of the tube was aspirated through a needle 16 cm. long into a sterile 10 cc. syringe, with due care that the needle point did not approach the side of the tube or extend into its slanting lower portion. A different syringe and needle was used with each tube, and the needle was disconnected while still immersed. The total fluid thus procured after the final centrifugation was not more than one-eighth to one-sixteenth of the original quantity; and hence centrifugation was begun with 100 to 200 cc. The ultimate material, searched in thick layer with the microscope, appeared free from cells, but showed particles close to the limit of visibility and very occasional fatty fragments of protoplasm or refractile granules having the same specific gravity as the fluid. The virus percentages or dilutions given in the protocols are calculated in terms of weight of infected tissue and cc. of extracting fluid.

In a few experiments the pellucid, supernatant fluid from cultures of embryo tissue infected with vaccinia was utilized as the virus suspension. When it had been "de-celled" in the way just described, it proved highly infective yet appeared almost free from particles. Sometimes a vaccinia supernatant was mixed with an extract of testicular tissue prior to decelling. None of the virus suspensions showed bacteria in stained smears.¹

The neutralizing sera were procured from rabbits recently recovered from vaccinia, and from others that had been repeatedly inoculated with the fibroma virus after disappearance of an initial growth. While the Shope fibroma is still growing the blood becomes capable of neutralizing the virus (8), but a first reinoculation not infrequently gives rise to a small nodule. Hence the need for further reinoculations with material of known activity. Bleeding for serum was done only when these had yielded negative results. The sera were ordinarily procured 2 or 3 days prior to use,—to ensure the presence of alexin; and they were centrifugalized several times at high speed to remove any cells. Many of our experiments were so arranged as to demonstrate the neutralizing capacity of the antisera used; but when this was not the case separate tests were done to make sure of it.

¹ Virus suspension as such mixed in equal amount with gel. Ty. sometimes gave rise to larger lesions, and in other experiments to smaller ones, than did cell materials exposed to more considerable amounts of the same suspension and repeatedly washed thereafter. In one uncharted test with vaccinia the virus as such caused no lesions, whereas the cells carrying it yielded large ones.

The Initial Association of Cells and Viruses

Do viruses become fixed upon the cells of susceptible animals, and if so, under what conditions? Are viruses protected by cells? To obtain answers to these questions living and dead cells were exposed to a virus suspension, and, after they had been washed, submitted to neutralizing sera, and repeatedly washed yet again, they were inoculated into rabbits.

General Technique.—Portions of cell suspension ($\frac{1}{2}$ or 1 cc.) were mixed at room temperature with a decelled virus suspension (1 to 3 cc.); after various intervals gel. Ty. was added to a total of 45 cc.; the cells were recovered by brief, slow spinning in flat-bottomed tubes; washed once more with 45 cc. of gel. Ty.; made to $\frac{1}{2}$ or 1 cc. with gel. Ty.; and placed in a large excess of antiserum neutralizing for the virus to which they had been exposed. To keep them suspended the container was slowly rotated on its long axis, during incubation at 37°C. Said container was a tube 1.5 cm. in internal diameter and 6.5 cm. long when stoppered. A bubble remained between stopper and serum when 10 cc. of fluid had been introduced, and the change in position of this bubble as the tube turned over kept the fluid stirred. The several tubes of each experiment were held with rubber bands against a square board fixed vertically at right angles to the shaft of a small motor making $3\frac{1}{2}$ revolutions per minute, in an incubator at 37°C. The control mixtures in normal serum or gel. Ty. were also turned or stood at room temperature. Afterwards all of the specimens were made to 45 cc. with gel. Ty., and the cells were collected by slow centrifugation, washed again in 45 cc. gel. Ty., and resuspended in 0.8 cc. or 1.0 cc., according as inoculation material for three or four rabbits was required. 0.2 cc. of each inoculum was injected into the shaved skin of the side, always by the same operator, at the same approximate level, and, as a rule, after all of the suspensions had been assembled. The injections were so arranged that those materials which supposedly would give the largest lesions remained *in vitro* the longest time; and when there were many inocula, those of which negative results were expected were alternated in situation with others of positive promise. The lesions near the groin tended to be largest. Hence the site of each material was varied from animal to animal, and, when possible, each control inoculation was made at the same situation relative to the groin as the corresponding experimental one on the opposite side. The lesions never became confluent. They were measured and charted from day to day. At each of the repeated washings the supernatant was drawn off as completely as possible, and at each resuspension the cells were distributed thoroughly. The centrifugings were done at a definite, slow rate. To prevent disturbance of the sediment in the flat-bottomed 50 cc. tubes it proved necessary to throw off the centrifuge brushes before the centrifuge stopped, and do the last braking with

the finger. The final inocula shimmered slightly with cells. In one instance in which they were unusually abundant, producing a definite cloudiness, an average of 610 per c.mm. was found on count.

Each experiment, from digestion to inoculation, was done in 1 day, and in each some of the test materials gave rise to lesions. Their size and character were recorded daily until retrogression began. In the charts a broken line indicates a more or less well defined, ruddy thickening of the skin, that is to say either a beginning lesion or an abortive one. Characteristic Shope tumors and vaccinia lesions are recorded in solid black, with hatching where there is vesiculation or necrosis. The charts are carried up to the beginning of retrogression only in the case of vaccinia, and through the first days of growth in that of the Shope fibroma.


































































































































































































































































































Comment on the Individual Experiments.—The findings were essentially the same with the Shope virus as with vaccinia. Charts 1-4 record them for two experiments with each virus. Certain points relating to each will be commented upon before they are submitted to analysis as a group. A number of less comprehensive tests were made which yielded corroboratory findings.

Experiment 1 (Chart 1) was planned to disclose whether Shope virus becomes associated with living and killed cells respectively, and whether in such association it is protected against the action of immune serum.

Living and killed cells in suspension were exposed to the virus, and washed and turned at 37° with immune serum, normal serum, and gel. Ty. respectively. The effects of the sera and of gel. Ty. respectively on the virus material as such were tested by injecting into the skin of the upper back 0.2 cc. of fresh mixtures in equal parts. The resulting lesions are not charted because of their exceptional situation. The virus-gel. Ty. mixture gave rise to lesions in all three rabbits, the mixture with normal serum in two, but that with immune serum in none.

Experiment 2 (Chart 2), also with the Shope virus, besides covering much of the ground of Experiment 1, was designed to test whether antibodies become fixed on killed cells subjected to immune serum and are carried over with them into the host, there to neutralize virus introduced at the same time.

Living cells submitted to virus and, after washing, to immune serum in the usual way (to neutralize any free virus remaining), were mixed after further washings with equal portions of killed cells washed after exposure to immune serum or Tyrode only (Inocula E, I, F, J). The mixing was done just prior to injection, and the resulting suspensions were made to the same bulk as the ordinary inocula,

Procedures	Summary	Rab.	Days							
			3	5	6	7	8	9	11	13
1 cc. <u>Living</u> Cell Suspension	<u>Living</u> Cells Submitted to Shope Virus		 5 cm.							
(B) Mixed with 2 cc. 8% virus, left 15 min., made to 43 cc., washed 4 times, and suspended in immune serum for injection (Injection 6:15-6:17 p.m.)	Injected with immune serum		Negative							
(A) Treated like B except that finally suspended in gel.Ty.	Injected with gel.Ty.	3 2 4 1	       	       	       	       				
(5:54-5:57 p.m.)										
(E) Submitted to virus, washed twice, then rotated 45 min. with 9 cc. gel.Ty. and again washed twice	Rotated with gel.Ty.	3 2 4 1	       	       	       	       				
(5:50-5:52 p.m.)										
(D) Treated like E except that rotated with 9 cc. normal serum instead of gel.Ty.	Rotated with normal serum	3 2 4 1	       	       	       	       				
(5:46-5:49 p.m.)										
(C) Treated like E except that rotated with 9 cc. immune serum instead of gel. Ty.	Rotated with immune serum	3 2 4 1	       	       	       	       				
(5:42-5:45 p.m.)										
(F) Mixed with 42 cc. of 0.4% virus, at once washed twice, rotated with immune serum, and again washed twice	Submitted briefly to same amount of virus in dilute suspension; then rotated with immune serum	3 2 4 1	       	       	       	       				
(5:39-5:41 p.m.)										
1 Cc. <u>Heated</u> Cell Suspension	<u>Heated</u> Cells Submitted to Virus									
(J) Treated like E	Rotated with gel.Ty.	3 2 4 1	       	       	       	       				
(5:28-5:30 p.m.)										
(I) Treated like E except that rotated with 1 cc. normal serum plus 8 cc. gel Ty.	Rotated with 1:9 mixture of normal serum and gel. Ty.	3 2 4 1	       	       	       	       				
(5:25-5:27 p.m.)										
(H) Treated like D	Rotated with normal serum	3 2 4 1	       	       	       	       				
(5:22-5:24 p.m.)										
(G) Treated like C	Rotated with immune serum		Negative							
(5:18-5:21 p.m.)										
1 Cc. <u>Fixed</u> Cell Suspension	<u>Fixed</u> Cells Submitted to Virus									
(K) Treated like E except that rotated with 1 cc. normal serum plus 8 cc. gel Ty.	Rotated with 1:9 mixture of normal serum and gel. Ty.	3 2 4 1	       	       	       	       				
(5:14-5:16 p.m.)										
(L) Treated like E	Rotated with immune serum		Negative							
(5:13-5:15 p.m.)										


Procedures	Summary	Rab.	Days						
			4	5	6	7	8	9	10
1 cc. <u>Living</u> Cell Suspension	<u>Living</u> Cells								
K) Double portion, washed 4 times (Injection 5:41-5:43 p.m.)	As such (double portion)		Negative						
A) Mixed with 3 cc. of 10% virus, left 15 min., made to 45 cc., and washed 4 times (5:47-5:49 p.m.)	Submitted to Shope virus	2	●	●	●	●	●	○	?
		3	●	●	●	●	●	●	●
		1	○	○	●	●	●	●	●
		4	○	—	—	—	—	—	—
B) Submitted to virus like A, washed twice, rotated 50 min. with 8 cc. immune serum, and washed twice (6:49-6:52 p.m.)	Submitted to virus and then to immune serum	2	●	●	●	●	●	—	●
		3	+	○	●	●	●	●	●
		1	—	—	●	●	●	●	●
		4	—	—	○	●	●	●	—
F) Treated like B + 1 cc. <u>heated</u> cell suspension washed 4 times (6:12-6:14 p.m.)	Treated like B + <u>heated</u> cells	2	○	○	+	—	—	—	○
		3	—	—	●	●	●	●	○
		1	—	—	●	●	●	○	○
		4	—	—	—	+	○	?	—
E) Treated like B + 1 cc. <u>heated</u> cell suspension rotated 50 min. with 8 cc. immune serum and washed twice (6:35-6:39 p.m.)	Treated like B + <u>heated</u> cells submitted to im- mune serum	2	—	—	●	●	●	●	○
		3	—	—	—	●	○	○	○
		1	—	—	○	●	●	○	○
		4	—	—	—	—	+	○	○
J) Treated like B + 1 cc. <u>rayed</u> cell suspension washed 4 times (6:30-6:33 p.m.)	Treated like B + <u>rayed</u> cells	2	—	—	●	○	○	○	○
		3	—	—	●	●	●	●	○
		1	—	—	●	●	●	○	○
		4	—	—	—	—	●	●	●
I) Treated like B + 1 cc. <u>rayed</u> cell suspension rotated 50 min. with 8 cc. immune serum and washed twice (6:44-6:47 p.m.)	Treated like B + <u>rayed</u> cells submitted to im- mune serum	2	?	—	●	○	○	●	●
		3	○	○	●	●	●	●	●
		1	—	—	●	●	●	○	○
		4	—	—	—	—	○	○	?
1 Cc. <u>Heated</u> Cell Suspension	<u>Heated</u> Cells								
(D) Treated like A (6:00-6:03 p.m.)	Submitted to virus	2	○	○	●	●	●	○	○
		3	—	—	—	○	●	●	●
		1	—	—	○	○	●	●	—
		4	—	—	—	—	—	—	—
(C) Treated like B (6:56-6:59 p.m.)	Submitted to virus and then to immune serum	2	?	?	+	+	—	—	—
		3	—	—	—	—	—	—	—
		1	—	—	○	○	○	○	—
		4	—	—	—	—	—	—	—
1 Cc. <u>Rayed</u> Cell Suspension	<u>Rayed</u> cells								
(H) Treated like A (5:33-5:36 p.m.)	Submitted to virus	2	—	—	—	—	○	○	○
		3	—	—	—	—	○	○	○
		1	—	—	○	?	?	?	—
		4	—	—	—	—	—	—	—
(G) Treated like B (7:03-7:06 p.m.)	Submitted to virus and then to immune serum		Negative						

CHART 2

with result that they contained twice as many cells per cc. Many of the cells killed by raying (10 minutes) fragmented during the subsequent washings and were lost. The experiment was done in the summer, and the control specimen *A* stood with gel. Ty. at a room temperature of 92°F. until the time of inoculation.

The initial cell suspension showed occasional large diplococci deriving from the tryptic digest. Inoculation of double portions of the cell material, after four washings like those of the test portions, gave rise to no lesions. The virus suspension alone, in 0.2 cc. amounts, gave rise to large lesions save in Rabbit 4, a naturally resistant animal. These lesions are not charted.

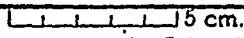
















































Experiment 3 (Chart 3) records the result of a nearly similar experiment with vaccinia.

To test whether serum antibodies were carried over into the host on killed cells, portions of these, washed after exposure to immune serum but not to virus, were mixed just prior to injection with other killed cells submitted to virus only and then washed (Inocula *D* and *II*), with appropriate controls. Two of the test rabbits showed generalized pocking on the 6th day after inoculation; and at this time of reduced resistance (9) lesions suddenly appeared where the heated cells turned with immune serum after exposure to virus had been injected. The other inoculations with killed materials submitted first to virus and then to immune serum yielded negative results, as had those of Charts 1 and 2. An occasional coccus was present in the initial cell suspension; but evidently it was non-pathogenic, since the inocula of living cells as such, and double portions of heated and rayed cells unexposed to virus, gave rise to no lesions, while cells exposed to virus gave typical ones.

In Experiment 4 (Chart 4) tests were made of the influence of time and of virus dilution on the fixation of vaccinia upon cells,—points already considered in Experiments 1 (Inocula *B* and *F*) and 3 (*B* and *C*). To see whether a preliminary exposure of living cells to immune serum would result in destruction of such virus as might become fixed on them later, a portion of them was turned with immune serum, washed, exposed to virus, and, after renewed washing, was turned with more immune serum (Inoculum *I*).

The cells killed by raying had been exposed for but 5 minutes and were well preserved.

Interpretation of the Findings.—The charts show that the inocula containing living cells repeatedly washed after exposure to virus gave rise regularly to lesions. The inoculation of living cells exposed to virus, rotated with immune serum, and washed also resulted in lesions, and so too did that of killed cells merely washed after exposure to virus. On the other hand killed cells that were exposed to virus, rotated with immune serum, and washed gave rise to no lesions or

Procedures	Summary	Rab.	Days						
			2	3	4	5	6	7	
<u>Living Cell Suspension</u>	<u>Living Cells</u>								
(L) Washed 4 times (Injection 5:03-5:05 p.m.)	As such		<u>Negative</u>						
(A) $\frac{1}{2}$ cc. mixed with 0.4 cc. $2\frac{3}{4}\%$ virus 15 min., thus diluting virus to 1.11% (or 1:90), made to 45 cc. with gel. Ty. and washed 4 times (5:14-5:16 p.m.)	Submitted to virus	1 2 4 3							
(B) $\frac{1}{2}$ cc. mixed with virus 15 min., diluted to 45 cc. and washed twice, mixed with 9 cc. immune serum, rotated 1 hr., and washed twice (5:11-5:13 p.m.)	Submitted to virus, then to immune serum	1 2 4 3							
(C) Same as B except 0.4 cc. virus was made to 45 cc. with gel. Ty. before addition of cells = cells submitted to 0.02% virus (1:5000) (5:08-5:10 p.m.)	Submitted to same amount of virus in dilute suspension, then to immune serum	1 2 4 3							
<u>Heated Cell Suspension</u>	<u>Heated Cells</u>		<u>Negative</u>						
(G) Double portion (1 cc.) washed 4 times (5:19-5:21 p.m.)	Double portion as such		<u>Negative</u>						
(E) $\frac{1}{2}$ cc. treated like A (submitted to virus and washed 4 times) + $\frac{1}{2}$ cc. washed twice (5:28-5:30 p.m.)	$\frac{1}{2}$ cc. submitted to virus + $\frac{1}{2}$ cc. as such	1 2 4 3							
(D) $\frac{1}{2}$ cc. treated like A + $\frac{1}{2}$ cc. rotated 1 hr. with immune serum and washed twice (5:32-5:34 p.m.)	$\frac{1}{2}$ cc. submitted to virus + $\frac{1}{2}$ cc. submitted to immune serum	1 2 4 3							
(F) $\frac{1}{2}$ cc. mixed with virus like A but washed only twice, then rotated 1 hr. with immune serum and washed twice + $\frac{1}{2}$ cc. washed twice (5:25-5:27 p.m.)	$\frac{1}{2}$ cc. submitted to virus, then immune serum + $\frac{1}{2}$ cc. as such	1 2 4 3							
<u>Rayed Cell Suspension</u>	<u>Rayed Cells</u>		<u>Negative</u>						
(K) Double portion washed 4 times†	Double portion as such		<u>Negative</u>						
(I) $\frac{1}{2}$ cc. treated like A + $\frac{1}{2}$ cc. washed twice (5:50-5:52 p.m.)	$\frac{1}{2}$ cc. submitted to virus + $\frac{1}{2}$ cc. as such	1 2 4 3							
(H) $\frac{1}{2}$ cc. treated like A + $\frac{1}{2}$ cc. rotated 1 hr. with immune serum and washed twice (5:46-5:48 p.m.)	$\frac{1}{2}$ cc. submitted to virus + $\frac{1}{2}$ cc. submitted to immune serum	1 2 4 3							
(J) $\frac{1}{2}$ cc. mixed with virus like A but washed only twice, then rotated 1 hr. with immune serum and washed twice (5:44-5:46 p.m.)	$\frac{1}{2}$ cc. submitted to virus, then immune serum + $\frac{1}{2}$ cc. as such		<u>Negative</u>						

* Generalized pocking now.

† Injection 5:40-5:42 p.m.

Procedures	Summary	Rab.	2	3	4	5	7
$\frac{1}{2}$ Cc. <u>Living</u> Cell Suspension	<u>Living</u> Cells		1 1 1 1 1 5 cm.				
(J) Washed 4 times	As such		Negative				
(A) Mixed with 1.0 cc. virus fluid,* left 1 hr., made to 45 cc., and washed 4 times	Submitted to virus	5					
		6					
		7					
		8					
(Injection 5:24-5:26 p.m.)							
(C) Mixed with virus fluid like A, then washed twice, rotated 1 hr. with 5 cc. immune serum, and washed twice	Submitted to virus, then to immune serum	5					
		6					
		7					
		8					
(5:11-5:13 p.m.)							
(B) Mixed with virus fluid only 2 min., then diluted to 45 cc. and treated like C	Submitted <i>briefly</i> to virus, then to immune serum	5					
		6					
		7					
		8					
(5:07-5:09 p.m.)							
(D) Mixed with a 1:40 dilution of virus fluid for 2 min., then treated like C and B	Submitted <i>briefly</i> to same amount of virus in dilute suspension, then to immune serum	5					
		6					
		7					
		8					
(5:04-5:06 p.m.)							
I) Rotated 1 hr. with 9 $\frac{1}{2}$ cc. immune serum, washed twice, and treated like C—6 washings in all	Submitted to immune serum, to virus, and to immune serum again	5					
		6					
		7					
		8					
(5:42-5:44 p.m.)							
$\frac{1}{2}$ Cc. <u>Heated</u> Cell Suspension	<u>Heated</u> Cells						
(H) Treated like A	Submitted to virus	5					
		6					
		7					
		8					
(5:32-5:34 p.m.)							
(F) Treated like C	Submitted to virus, then to immune serum	5					
		6					
		7					
		8					
(5:28-5:30 p.m.)							
$\frac{1}{2}$ Cc. <u>Kayed</u> Cell Suspension†	<u>Kayed</u> Cells						
(G) Treated like A	Submitted to virus	5					
		6					
		7					
		8					
(5:22-5:24 p.m.)							
(P) Treated like C	Submitted to virus, then to immune serum	5					
		6					
		7					
		8					
(5:20-5:22 p.m.)							

* Deoiled mixture of gel. Ty. extract from infected testicle and supernatant fluid from a culture of vaccinal tissue.

† Kaying was for only 5 minutes.

to but trifling ones. The experiments seemed to show that the viruses became fixed on both living and dead cells, and were carried through the washings with them. When now the living cells were exposed to immune serum such virus as they carried was not in the least affected, whereas that associated with killed cells underwent neutralization. Before these implications of the findings can be accepted some alternative possibilities must be considered.

Will a persistence of virus in the free state throughout the manipulations explain the results?

The four successive washings of the cells with gel. Ty. after exposure to virus should on calculation have diluted any free virus more than one million times. The steps taken to remove particles which might contain virus from suspensions of the latter have already been described. If the persistence into the final inoculum of such particles, or of suspended free virus, had been responsible for the lesions, the killed cell material exposed to virus and then to immune serum should have produced lesions, since living materials similarly treated did so.

Some tissue extracts are known to enhance the activity of vaccinia injected with them (Reynals factor). May not a failure of killed cells to enhance the lesions explain, in part at least, the differing results with them? This can scarcely be since the controls with killed cells yielded large lesions (Charts 3 and 4).

Will the presence of neutralizing serum principles in the inocula explain the differences found?

The washings of the cells exposed to serum diluted the latter to between 1-1500 and 1-3000 in the inocula. On comparing the lesions from Inocula *E* and *I* of Chart 2 with *F* and *J*, as also *D* and *H* with *E* and *I* of Chart 3, it will be seen that when material containing virus thus diluted was mixed, just prior to inoculation, with other material carrying virus in minimal amount, as good lesions were obtained as with similar cell mixtures from which serum was absent. Evidently the serum as such cannot have reached the inocula in effective amount. Nor, as the tests show, can neutralizing serum principles have been carried on the dead cells into the animal, there to neutralize virus introduced at the same time.

From all this it is plain that the lesions produced by inoculation of the cells must have resulted from virus fixed upon them. This fixation was so firm as to withstand agitation of the cells with serum during a considerable period, and repeated pipettings with large quantities of wash fluid, together with as many as six changes of the latter. So small was the cell content of the inocula that they appeared almost pellucid. Yet they gave rise to lesions save when nearly all the cells

had been lost, as in some instances when treatments with ultraviolet rays had rendered them friable (Chart 2, *H*) or when they had been killed before they were submitted to neutralizing serum. The differences in the results with living and killed cells exposed to such serum cannot be laid to a carrying over into the final inocula of the serum as such.

The fixation of virus on the cells took place rapidly.

In Experiment 1 (Chart 1), 1 cc. of living cell suspension was mixed with 42 cc. of a 1 in 21 dilution of 8 per cent Shope virus (0.4 per cent virus) in a tube already balanced for centrifugation; and this was forthwith spun at low speed for 10 minutes with another tube in which the same quantity of cell suspension had been standing with 1 cc. of 8 per cent virus for 15 minutes prior to dilution to 0.4 per cent just before the spinning. After the usual two washings both cell portions were exposed to immune serum and washed twice again. They gave identical results on inoculation (Chart 1, *C* and *F*). In Experiment 4 cells that had stood with virus for 2 minutes prior to washing and exposure to immune serum gave lesions as large as those that had stood for an hour (Chart 4, *B* and *C*).

The virus suspensions were free from particles that could be readily thrown down with the centrifuge. Nevertheless several tests were made to see whether fixation occurred while the cells were in suspension, or whether it was due to virus caught between the tube bottom and the sedimented cells with result that it became attached to them. In the latter case cells that had stood with a dilute virus suspension should have yielded as large lesions as when they had stood with virus not diluted until just before the tubes were spun. In one instance just discussed the two specimens did give identical results. In two other tests, however, (Chart 3, *B* and *C*; Chart 4, *B* and *D*) the lesions from cells that had stood with virus already diluted were the smaller. The conditions did not permit of great virus dilution, and no more considerable differences in result could have been expected than were encountered.

While the test mixtures proper were rotating with serum in the incubator, the control of living cells merely exposed to virus and washed was in most instances let stand at room temperature, the object being to minimize the deterioration of virus that takes place in salt solutions. In Experiment 1 (Chart 1, *A* and *E*) the effects of these procedures on the Shope virus were compared and no difference was found. (See also Chart 7, *A* and *B*.) In the case of vaccinia, however, the control lesions were decidedly the larger. No great difference was observed in experiments yet to be described, with cells long exposed to vaccinia in tissue cultures (Chart 5, *A* and *D*; Chart 9, *B* and *C*). It seems probable that in the present instances of brief exposure to the virus, some of it was so loosely fixed as to have been still accessible to the immune serum, or else it was dislodged during the turning. Exposure of the virus to 37° during the period of turning cannot have been

an important factor, since 53° for 15 minutes had almost no effect upon it (Charts 5 and 6, A and C).

The fixation of Shope virus upon killed cells exposed briefly thereto would appear from the lesions of Charts 1 and 2 to have been considerably less, or less firm, than upon living ones; but in the vaccinia experiment (of Chart 4) as well as in some with cells from cultures of both sorts (Charts 5, 6, 8, 9) the magnitude of the lesions indicated that almost or quite as much virus was carried by the dead material.

The adsorption of viruses upon inanimate matter is known to be much affected by the pH and other qualities of the fluid medium. The association of the Shope virus with the living cells upon which it had become fixed would appear, however, to have been wholly unaffected by incidental differences in the fluids used in our experiments. Lesions of the same magnitude were produced by virus-laden cells that had been rotated with immune serum, normal serum, and gel. Ty. respectively, or had been merely let stand with the latter (Chart 1). It seemed possible, however, that virus might come away from dead cells, as not from the living, during rotation with serum and be lost in the subsequent washings. The results with Inocula J, I, H, and G of Chart 1 bear upon this point. The virus-laden cells were rotated with gel. Ty., with normal serum as such or diluted, and with immune serum respectively. Only in the case of the last did the inocula fail to give lesions. In Experiment 10 (Chart 9) water-killed cells rotated in a dilute mixture of immune serum and gel. Ty. gave rise to lesions almost as large as those from material thus killed and merely washed at room temperature and injected forthwith. The cells turned with undiluted immune serum on the other hand yielded lesions which were relatively insignificant.

It seems clear from the results as a whole that vaccinia and the Shope virus become quickly, firmly, and abundantly fixed upon individual cells derived from cultures of rabbit embryo. Fixation takes place on both living and killed cells. Exposure *in vitro* to neutralizing serum does not affect the virus when the cells with which it is associated are alive, as is proven by the size of the lesions resulting from inoculation of the material. If they are dead, though, when exposed to the serum, the material gives rise to no lesions or to negligible ones. It was repeatedly noted that no lesions develop when living cells

carrying the viruses are injected together with immune serum. This fact will be considered further in the light of experiments now to be described.

The Association of Viruses with the Cells of Tissue Cultures

Vaccinia can be demonstrated in abundance in the fluid of tissue cultures in which it is under propagation. Does there exist, in addition, a fixation of the virus in or upon the growing tissue cells? If so, are the latter capable of protecting the virus from neutralization with serum? These points were investigated by liberating cells from tissue cultures of vaccinia, and subjecting them to experiments of the sort just described. Similar tests were made with cells from cultures of the Shope tumor.

The Vaccinial Cultures.—Cultures of rabbit embryo tissue were made by our usual technique except that just prior to implantation one part of vaccinial material was added to six parts of tissue suspension. Ordinarily a 10 per cent centrifugalized extract in Tyrode of glycerinated or fresh testicular material on the 3rd to 5th day of infection was added; but in occasional instances the supernatant fluid from cultures infected with such material was utilized to start new ones which were employed for the experiment proper. As a rule 8–10 flask cultures were prepared at one time, together with others containing embryo tissue only. They were washed with gel. Ty. every 2–3 days and utilized after 5 to 7 days in all. Injections of the decelled supernatant fluid from the cultures resulted in large vaccinial lesions, and inoculation of the cells liberated with trypsin regularly showed the presence of virus in quantity. The inoculated cultures grew far less well than did the uninoculated controls; the tryptic digest was more clouded with debris; the living cells that had been freed were mostly fatty; and great numbers of refractile bodies were present having some resemblance to those liberated by trypsin from fowl pox tissue (10).

The bodies mentioned were 10 to 50 μ in diameter,—not larger than some of the associated cells. They had the form of slightly irregular, pale buff spheres, with an ill-defined, coarse facetting. The small ones were frequently intracellular, but the large appeared free. They were present in quantity in the supernatant fluid drawn off after the first slow spinning of 5 to 7 day old vaccinial cultures, though some were entangled and carried down with the tissue debris. Rapid centrifugation did not bring them either to the surface or the bottom,—so closely did their specific gravity approximate that of the fluid. Unlike the inclusions of fowl pox studied by Goodpasture and Woodruff (11) they did not disintegrate when subjected to strong trypsin in the presence of sodium bicarbonate, nor did they swell on exposure to distilled water. Like such inclusions, however,

they ruptured at the moment when water evaporated from about them. Pressure on the cover-glass also caused them to rupture, forcing out a single, round, highly refractile globule from a thin, transparent, wrinkly sac, probably the remains of an enveloping cell. The globules thus obtained looked precisely like the fatty ones that could be pressed out of degenerating culture cells, and they readily dissolved in alcohol and ether. When dried bodies were subjected to these reagents there was left only the sac. Morosow's stain colored them a homogeneous yellow like the cell fat, not black like the particles described by Paschen. Altogether the evidence indicates that we were dealing with the metabolic products of cells injured by the virus, a view supported by the very occasional recovery of elements of identical appearance and behavior from cultures uninfected with vaccinia.

Character of the Experiments.—The methods were those already described. The digests of eight to ten cultures were pooled, and the freed cells were separated from the tissue fragments by filtration, twice washed, and suspended for test. Debris was practically absent from the ultimate suspensions, and neutral red showed nearly all of its cells to be alive. Heating at 53° for 15 minutes was ordinarily used to kill them. The heat affected the virus little if at all (Charts 5 and 6).






































































































Tests were made to see if the washed, living and killed cells carried virus, and whether they protected it from the influence of neutralizing serum of proven potency.

Comment on the Findings.—The experiments yielded consistent results. Charts 5 and 6 of Experiments 5 and 6 record their outcome in two typical instances.


















































































Living culture cells that were twice washed with 45 cc. of gel. Ty.,—in addition to the several washings incident to preparation of the suspension,—regularly gave rise to characteristic vaccinal lesions. When they were also exposed to a neutralizing serum the lesions were almost as large; and so too when they were killed with heat and then washed. Cells subjected to immune serum, washed, and killed with heat, produced lesions that were slightly less marked, though characteristic,—as if the slightly detrimental effects of heating and of immune serum had been added together. In pronounced contrast were the almost completely negative results with cells killed with heat prior to exposure to serum. Either they caused no lesions or small nodular ones appearing late.

The persistence of free virus and of neutralizing antibodies, as influencing the outcome of the tests, can be excluded on the same grounds as in the experiments already described (*q.v.*), and so too can the possibility that serum antibodies became fixed on the dead cells and were carried over into the host, there to neutralize the virus introduced with them.

The experiments show that vaccine virus was carried by the culture cells, and was so closely associated with them as not to be removed by washings and pipettings, even after the cells had been killed by heat.

Procedures	Summary	Rab.	Days				
			2	3	4	5	7
<i>1 Cc. Cell Suspension from Vaccinial Cultures</i>	<i>Vaccinial Cells</i>		 5 cm.				
(B) Washed twice and suspended in immune serum for injection* (Injected 3.52-3.55 p.m.)	Injected with immune serum*		Negative throughout				
(A) Washed twice and suspended in gel.Ty. for injection	Injected with gel.Ty.	2					
		1					
		4					
		3					
(3:58-4.01 p.m.)							
(C) Heated at 53° for 15 min., washed twice, and injected	Killed with heat and washed	2					
		1					
		4					
		3					
(3:42-3:45 p.m.)							
(D) Rotated 1 hr. with 9 cc. of immune serum and washed twice	Cells rotated with immune serum	2					
		1					
		4					
		3					
(3:35-3:38 p.m.)							
(E) Treated like D and heated at 53° for 15 min. just prior to injection	Rotated with immune serum and killed with heat	2					
		1					
		4					
		3					
(3:29-3:31 p.m.)							
(F) Heated as was C, rotated in 9 cc. immune serum and washed twice	Cells killed with heat and rotated with immune serum	2					
		1					
		4					
		3					
(3:18-3:23 p.m.)							

* All other final suspensions were made in gel. Ty.

Procedures	Summary	Rab.	Days			
			2	3	4	6
<i>1 Cc. Cell Suspension from Vaccinial Cultures</i>	<i>Vaccinial Cells</i>					
(B) Washed twice and suspended in immune serum for injection*	Injected with immune serum*	2 4 3 1	   	   	   	   
(A) Washed twice and suspended in gel.Ty. for injection (Injected 4:34-4:37 p.m.)	Injected with gel.Ty.	2 4 3 1	   	   	   	   
(C) Heated at 53° for 15 min. and washed twice (4:27-4:30 p.m.)	Killed with heat and washed	2 4 3 1	   	   	   	   
(D) Rotated 1 hr. with 9 cc. immune serum, and washed twice (4:23-4:26 p.m.)	Rotated with immune serum	2 4 3 1	   	   	   	   
(E) Treated like D and heated at 53° for 15 min. just prior to injection (4:12-4:15 p.m.)	Rotated with immune serum and killed with heat	2 4 3 1	   	   	   	   
(F) Heated as was C, rotated with 9 cc. immune serum, and washed twice (4:04-4:10 p.m.)	Killed with heat and rotated with immune serum	2 4 3 1	 	 	 	

* All other final suspensions were made with gel. Ty.

The immune serum failed to affect the virus associated with living cells, whereas that associated with killed cells was neutralized by it.

The Cultures Containing Shope Virus.—To obtain the Shope virus in cultures the hashed tissue of young, growing fibromas was cut up and implanted precisely as was rabbit embryo in the work with vaccinia. Large, individual cells moved out early from the tissue bits and occasionally entered the supernatant fluid in considerable number. They segregated neutral red promptly in granules of highly various size and had the general appearance of clasmatocytes. After the first 2 or 3 days elements of fibroblastic type began to extend out in strands, which were fairly numerous by the 6th or 7th day. Only when this had happened were the cultures digested.

Character of the Experiments.—Some early tests were directed to determining whether the virus responsible for the fibroma was present in the supernatant fluid of the cultures and in the fluid portion of the tryptic digest. As a rule these materials were decelled and 0.2 cc. was injected.

The suspensions of culture cells were procured as in the case of vaccinia. Heating to 53° for 15 minutes, or raying for 5 minutes rendered the virus inactive, and hence could not be utilized to kill the cells. Water was employed instead. The cells from standard portions of well washed suspension were thrown down, resuspended in 2 or more cc. of twice distilled water, and allowed to stand for 56–60 minutes at room temperature, the controls meantime standing in the same amount of gel. Ty. As result the cells became greatly swollen, and the staining of the nuclei with neutral red showed that they were dead. They did not completely return to normal size when isotonicity was restored by washing them in an excess of ordinary gel. Ty. or by adding in appropriate quantity "triple" Tyrode,—a solution containing the salts of Tyrode in triple strength; and they tended to fragment when washed, necessitating longer centrifugations than usual to prevent loss. The gel. Ty. controls were centrifuged at the same time. Each of the ultimate materials was made to a bulk of 0.7–0.75 cc., and three rabbits were injected with 0.2 cc. of this.

Comment on the Findings.—Chart 7 portrays the results of an orienting experiment, Experiment 7, typical of several of the sort. The supernatant culture fluid contained living cells of makrophage type in fair number. It was inoculated as such, whereas the fluid portion of the tryptic digest was decelled prior to inoculation. The suspension of washed culture cells was divided into three portions of 1.2 cc., of which one was let stand in gel. Ty. at room temperature, while the other two were rotated with gel. Ty. and with immune serum respectively for 2 hours.

The three cell materials all gave rise after approximately the same time to characteristic tumors which grew at the same general rate. The tryptic digest fluid on the other hand caused a lesion in but one animal, and this consisted of several small, shotty growths appearing late and coalescing. The supernatant from the cultures gave rise late to more numerous growths of the same sort.

They were scattered over an area of several cm. on the rabbit's side, but have been charted close together to save space, a procedure employed in some of the other chartings also.

Procedures	Summary	Rab.	Days			
			7	9	14	23
<i>Cells from Shope Culture</i>	<i>Shope Culture Cells</i>		5 cm.			
(A) Suspended in 1.2 cc. gel. Locke's and let stand 2 hrs. at room temperature	Cells let stand at room temperature	3				
		2				+
		1				+
(B) Rotated 2 hrs. in 10 cc. gel. Locke's, twice washed, and made to 1.2 cc.	Cells rotated with gel. Locke's	3	-	-		
		2				+
		1			**	-
(C) Rotated 2 hrs. in 10 cc. immune serum, twice washed, and made to 1.2 cc.	Cells rotated with immune serum	3				
		2				+
		1			-	-
(D) Decelled fluid from tryptic digest	Decelled fluid from tryptic digest	3	-	-		
		2	-	-	-	-
		1	-	-	-	-
(E) Supernatant fluid from cultures*	Supernatant* from cultures	3	-	-		
		2	-	-	-	-
		1	-			-

* Contained some inwandered cells.

** Measured on 8th day and excised.

CHART 7

Experiments 8 and 9 (Chart 8) were more extensive. The supernatant from the cultures was decelled in both cases. In each it had been only 2 days on a

culture 4 days old. It gave rise late to nodules that tended to be small, scattered, and shotty, as did also the decelled tryptic supernatant. In Experiment 9 this contained some bacteria, evidently from a colony overlooked in the examination

Procedures	Summary	Rab.	Experiment 8 Days			Rab.	Experiment 9 Days		
			3	5	9		4	6	8
<i>Cells from Shope Culture</i>	<i>Shope Culture Cells</i>		1 5 cm				1 5 cm		
(A) Made to 0.7 cc. with gel. Ty.* and allowed to stand 45 min. at room temperature	Allowed to stand in gel. Ty.	3				1			
		2				3			
		1				2			
(B) Placed in 2 cc. H ₂ O for 45 min., then in excess of gel. Ty., and washed once	Killed with H ₂ O and washed	3				1			
		2				3			
		1				2			
(C) After 45 minutes in 2 cc. gel. Ty. 8 cc. immune serum added, with rotation 1 hr.; then washed twice and treated like B	Rotated with immune serum, killed with H ₂ O, and washed	3				1			
		2				3			
		1				2			
(D) After 45 minutes in 2 cc. H ₂ O 8 cc. immune serum added, with rotation 1 hr. and 2 washings	Killed with H ₂ O, rotated with immune serum, and washed	3				1			
		2				3			
		1				2			
(E) Decelled fluid from tryptic digest mixed in equal part with gel. Ty.	Decelled fluid from tryptic digest	3				1			
		2				3			
		1				2			
(F) Decelled supernatant culture fluid	Decelled supernatant culture fluid	3				1			
		2				3			
		1				2			
(G) Cells from supernatant culture fluid	Cells from supernatant culture fluid	3				1			
		2				3			
		1				2			

* Ultimate amount of all inocula in Experiment 8, 0.7 cc., in Experiment 9, 0.75 cc.

CHART 8

of the cultures; but these were non-pathogenic as the outcome of the inoculations attested. The cells collected from the supernatant of Experiment 9 during the decelling were relatively few and of makrophage type; yet they gave rise, after two washings, to discrete, small growths like the larger ones resulting from the culture cells proper. In each experiment one portion of the latter was allowed to stand

Procedures	Summary	Rab.	Experiment 10 Days		
			3	4	5
<i>1 Cc. Cell Suspension from Vaccinal Cultures</i>	<i>Vaccinal Culture Cells</i>				
(A) Washed twice after standing 45 min. in gel.Ty.	Washed after standing	2			
		1			
		3			
(Injected 4:25-4:27 p.m.)					
(B) Killed with H ₂ O,* made isotonic, and washed twice	Killed with H ₂ O and washed	2			
		1			
		3			
(4:28-4:30 p.m.)					
(C) Rotated 1 hr. with 8½ cc. immune serum washed twice, killed with H ₂ O, made isotonic, and again washed twice	Rotated with immune serum and killed with H ₂ O	2			
		1			
		3			
(7:03-7:05 p.m.)					
(D) Killed with H ₂ O, made isotonic, rotated 1 hr. with 2 cc. immune serum and 6½ cc. gel.Ty.	Killed with H ₂ O and rotated with dilute immune serum	2			
		1			
		3			
(5:52-5:54 p.m.)					
(E) Killed with H ₂ O, made isotonic, rotated 1 hr. with 8½ cc. immune serum, and washed twice	Killed with water and rotated with immune serum	2			
		1			
		3			
(5:47-5:48 p.m.)					

* Cells thrown down, suspended in excess of twice distilled water (3.2 cc.), let stand 45 minutes at room temperature, and brought to isotonicity with triple strength p.Ty.

in gel. Ty. after washing, while another was submitted to water and later washed in an excess of gel. Ty. to restore tonicity. These two specimens, the one of living, the other of killed and somewhat fragmented cells, gave rise to lesions of practically identical size, ruddy projecting growths that appeared in 3 or 4 days, grew vigorously and were soon capped by a broad vesicle. The third cell portion, submitted to immune serum of high potency and then washed, water-killed, and washed again, yielded only slightly smaller lesions, appearing somewhat more slowly. In marked contrast were the findings when the cells had been killed with water prior to contact with serum. This material was not washed so often as that just mentioned and it reached the final suspension in at least as great quantity; yet it gave rise to poor growths that appeared late.

The importance of the bodily condition of the test animal on the development of Shope lesions is illustrated in Chart 8. One of the rabbits (No. 2 of Experiment 9) developed diarrhea shortly after inoculation, and losing weight rapidly, died on the 10th day. Only the more virulent of the inocula caused recognizable lesions in this animal; and they remained so small as to afford few contrasts. Rabbit 1 also afforded few contrasts, but that was because nearly every inoculum caused a large growth.

In Experiment 10, one of several in a group apart, water was used to kill vaccinia culture cells, and isotonicity was restored by adding triple Tyrode prior to the addition of serum. As Chart 9 shows, the material killed with water and washed gave rise to smaller lesions than did that which had merely stood in gel. Ty. The lesions were pronounced and characteristic, however. Material turned with immune serum, washed, killed, and washed twice again yielded lesions nearly as large, whereas that killed prior to rotation with immune serum (with omission of two washings) had but little pathogenic effect.

The individual cells procured by digestion from cultures of the Shope fibroma carried the causative virus. Indeed it was almost wholly localized to the cells, very little being free in the supernatant culture fluid or even in the decelled tryptic digest (Charts 7 and 8). Repeated washing of the living culture cells, followed by exposure to potent immune serum *in vitro*, failed to remove or neutralize the virus associated with them. When killed cells were exposed to the serum, however, the virus was rendered ineffective (Figs. 2 and 3). Repeated washings in gel. Ty. had no evident effect on the virus associated with the killed cells.

The lesions produced by the culture cells and the supernatant fluids respectively were significantly different, although the areas of skin infiltrated by the injection were of the same size. When either living or dead cells had been introduced the tumor developed as a solitary, spherical or discoid mass at the point

of injection, whereas after the introduction of decelled, supernatant fluid the growths were often multiple,—appearing as shotty nodules scattered more especially in the direction of lymph drainage (Figs. 2 and 3). Evidently the injected cells were retained at the injection point, together with the virus fixed upon them, whereas virus free in the supernatant fluid tended to be more widely distributed.

So soon did Shope tumors appear in certain instances after the injection of the living cells as to suggest that some of these had survived and proliferated; but the water-killed cells yielded tumors nearly as promptly (Chart 8), indicating that transplantation had played little part in the results.

In the case of vaccinia the tests do not enable one to say whether the demonstrated association of cells and virus is obligatory for the latter; for during cultivation it may have become fixed secondarily on the cells, as happens when the two are left together briefly *in vitro* (Charts 3 and 4). True, the lesions produced by washed, living, vaccinia culture cells submitted to immune serum and washed again were far larger than those resulting from the injection of rabbit embryo cells exposed but briefly to virus and then to immune serum; and in tests of the latter sort the serum cut down the size of the lesions considerably. But this may have resulted merely from a more abundant or firmer fixation upon the cells derived from vaccinia cultures. The findings with the Shope virus point to an obligate association of it with the cells.

DISCUSSION

The experiments prove that viruses rapidly become fixed upon individual living cells. Can the fixation be due to phagocytosis? In fowl plague and cattle plague the virus present in the blood stream is largely associated with the white corpuscles (12); and a similar association of vaccinia with leukocytes is demonstrable in rabbits at the height of infection (13). In these instances the phenomenon may have resulted from phagocytosis not as yet followed by virus death; for Douglas and Smith (14) and Fairbrother (15) have produced evidence that the blood leukocytes destroy vaccinia. But in our experiments the fixation upon cells was practically immediate at room temperature, and the quantity fixed was large, judging from the lesions caused. The virus-containing suspensions themselves were the only possible source of opsonins, and they had undergone a several thousand-fold dilution with Tyrode solution.

Many workers have supposed that the virus of fowl plague becomes fixed upon the red corpuscles; but Todd's recent work makes this unlikely (16). Some have reported that the virus of foot and mouth

disease can become fixed upon red cells, while others deny this. Several authors have demonstrated that viruses collect out of suspension upon tissue fragments, notably Kraus, von Eisler, and Fukuhara (17), and adsorption upon charcoal, kaolin, aluminum hydroxide, and other substances is known to take place rapidly. In most of the experiments the viruses remained firmly fixed to the cells after the latter had been killed; yet one cannot infer that the conditions of fixation remained the same. Krueger (18), studying the association of phage with bacteria of susceptible sort, has found that when the latter are alive phage is "distributed in a manner typical of numerous materials soluble in both phases of a two-phase system." The fixation upon dead bacteria, on the other hand, is of adsorptive type. The strikingly different findings in our tests with living and dead cells carrying a virus and exposed to neutralizing serum is indicative of a great difference in the conditions. Living cells protected the virus from the influence of the serum whereas dead cells did not.

Andrewes (19) has found that immune serum injected into the skin 5 minutes after an inoculation of vaccine virus into the same spot fails to prevent the development of a lesion. If instead the serum is given 5 minutes beforehand no lesion appears. ("The serum has thus acted in five minutes.") The suggestion has been made by both Andrewes and Fairbrother that in the skin the immune serum acts on the local tissue cells as well as on the virus. In our experiments cells were present. Fairbrother (20) proved by intracerebral inoculations that vaccinia virus is neutralized by standing 4 hours *in vitro* with immune serum and that there is some neutralization in 1 hour. In the tests of the present work an enormous quantity of serum was employed as compared with the amount of virus fixed upon the cells. Antibodies for viruses are readily adsorbed out of serum on collodion particles and diatomaceous earth (21). But certainly no effective fixation occurred on either the living or dead cells of our tests despite excellent opportunities.

In a previous paper from this laboratory (22) the fact has been brought out that living cells protect erythrocytes and bacteria situated within them from hemolysins and the bactericidal principles of homologous serum, as well as from the lethal effects of potassium ferrocyanide. Dead cells are readily penetrated on the other hand by the agents mentioned and these exert their destructive influence. One is tempted to suppose that the viruses used in the present experiments, after becoming attached in some way to living cells, are taken into the

latter, and owe their persistence in active state to an intracellular situation. But the data do not justify this supposition. They prove only that the protection of the viruses is in some way dependent upon cell life. The maintenance of a special state of affairs at or near the cell surface might suffice for protection.

Perdrau and Todd (23) found that tissue freshly procured in suspension from organs infected with viruses, shielded the latter from photodynamic inactivation by methylene blue, whereas after the tissue had been submitted to glycerine no protection was demonstrable. Normal tissues exposed *in vitro* to the viruses and then to the photodynamic influence failed to produce lesions on inoculation,—whence Perdrau and Todd concluded that a penetration of the viruses into the interior of the cells was essential to their protection, and that it had not had time to occur in the tests last mentioned. Our experiments make it seem likely that a fixation of Perdrau and Todd's viruses, one of which was vaccinia, took place on the normal tissues, but that such protection as these may have exerted did not suffice against the photodynamic activity. The authors stated that fragments of tissue as well as separate cells were present in the suspensions tested; and the protection they noted in the case of materials prepared from organs already containing virus may have been due to the situation within tissue bits. The inactivation of viruses by methylene blue is dependent upon the presence of oxygen; and Perdrau and Todd attribute the protection of the viruses within infected tissue to the maintenance by the cells of a special oxidation-reduction potential.

The suggestion has been put forward in a previous paper (24) that a protection of pathogenic bacteria by living cells will explain the obduracy of certain diseases, as *e.g.* tuberculosis, leprosy, and gonorrhea. There is much to indicate that this is the case in some virus diseases as well. Smith (25) has been able to recover vaccine virus from blood leukocytes and those of the peritoneal cavity at intervals up to the 8th or 9th day of infection, although antibodies had been in circulation since the 2nd or 3rd day; and Douglas, Smith, and Price (26) have demonstrated its presence in some of the organs of rabbits recovered from vaccinia inoculated 41 days previously. Olitsky and Long (27) have separated out the virus by electrophoresis from the testicles of immune rabbits so long after inoculation that they believe that it persists indefinitely within the body.

The behavior of growths due to viruses is largely referable to a protection of the latter from circulating immune principles. Two

sorts of resistance can be discriminated in fowls carrying Chicken Tumor I, against cells and causative agent respectively (28). The transplanted cells of this sarcoma will often give rise to tumors in fowls that are completely resistant to the agent itself, and these may progress rapidly and kill despite the presence in the blood of neutralizing antibodies for the agent (29). This is the case with other chicken tumors also (30). The development of the Shope rabbit papilloma, a virus-induced epithelial growth with the characters of a tumor (31), is attended by the appearance in circulation of principles effective against the virus as such (32). Yet the growth continues to proliferate and its cells can be successfully transferred within the host, although the latter is now completely resistant to the virus present in association with the cells and responsible for their multiplication.

When, in our experiments, immune serum was injected together with the virus-carrying, living cells no lesions developed. But vaccinia as a necrotizing virus, doubtless killed almost at once those elements with which it was introduced, thus exposing itself to the action of serum injected with it. Andrewes has shown that the antibodies of serum from animals immune to vaccinia remain localized for as long as 72 hours after intradermal injection, and capable of neutralizing virus introduced into the same spot during this period. The Shope virus induces cell multiplication; but the cells carrying it in our tests had been badly maltreated. That they did not long survive is indicated by the almost equally good lesions produced with water-killed cells.

The prompt fixation of viruses upon living cells, with protection of them afterwards, will go some distance to explain how viruses obtain a foothold in the host, and why serum treatment is so often unsatisfactory once infection is under way. Loss of protection when the virus-affected cells die in the period after antibodies have come into circulation may be the reason for recovery from virus diseases. Local vaccinal lesions evidently develop and progress by a multiplication of the virus in association with cells which, however, are soon killed by it. The resulting exposure of the virus to principles seeping into the lesion from the blood can deter but little the progress of the disease in the days before effective antibodies come into circulation. But when this happens one may suppose that the virus is neutralized

wherever it deprives itself of the protection of cells by killing them. Thus in due course the disease is overcome, though the virus itself may persist in occult form in association with cells that it does not kill. Conditions are somewhat different with the Shope fibroma. Shope reports that the growth continues to enlarge for some time after neutralizing antibodies for the virus have appeared in the blood. Possibly the cells affected by the virus ultimately degenerate and no longer protect the virus from serum antibodies of mounting potency. The histological changes in retrogressing Shope fibromas support this supposition.

Some features of the behavior of the Shope virus have been disclosed incidentally to the work. Like the agent causing Chicken Tumor I, the virus is relatively ineffective in sick or undernourished hosts, causing small growths or none at all. If the condition of the animal improves soon after the inoculation there may be a late appearance of fibromas. Young animals are the most favorable to the growth's enlargement, as in the case of many neoplasms. The virus, like that of Chicken Tumor I, is killed by slight heating, but unlike it is rapidly inactivated by ultraviolet light. In our experience adding kieselguhr to a Shope virus suspension does not increase and may interfere with its effectiveness to cause growths, whereas it greatly aids the agent producing Chicken Tumor I. The latter virus is regularly present in abundance in the supernatant fluid of cultures of the tumor tissue, whereas the fluid from cultures of the Shope fibroma is almost completely innocuous.

The injurious effects of vaccinia on the cells of tissue cultures in which the virus is under propagation seem not to have been observed before. Traub has recently reported that cell damage occurs when pseudorabies virus (32) is cultivated with tissue *in vitro*. The curious bodies appearing in vaccinia cultures merit further scrutiny.

SUMMARY

Methods were developed for a study of the relations existing between viruses and living cells. It was found that vaccinia and the virus causing the infectious fibroma of rabbits (Shope) rapidly become fixed upon tissue cells freed as individuals and submitted to virus in suspension. This happens whether the cells are alive or have been killed

with heat or ultraviolet light. The virus does not come away during agitation of the cells with Tyrode solution and repeated washings with large amounts of it. The exposure to neutralizing antisera of cells carrying virus fails to affect this latter significantly if the cells are alive, whereas if they are dead the activity of the virus is nullified. Cells freed as individuals from tissue cultures of vaccinia and the Shope tumor carry these viruses in abundance through repeated washings, and, if living, protect them from the influence of a neutralizing serum, whereas killed cells exert no such protection.

The findings would appear to throw light on the way in which viruses gain a foothold in the host; and they suggest reasons for the persistence of some viruses in recovered animals and for the unsatisfactory results of serum treatment instituted during the course of virus diseases.

The virus causing the Shope fibroma has been successfully maintained in cultures of the growth. It is closely associated with the cells, almost none being present in the culture fluid. Certain of its other attributes have been determined. Vaccinia greatly damages the cells of cultures of rabbit embryo in which it is under propagation.

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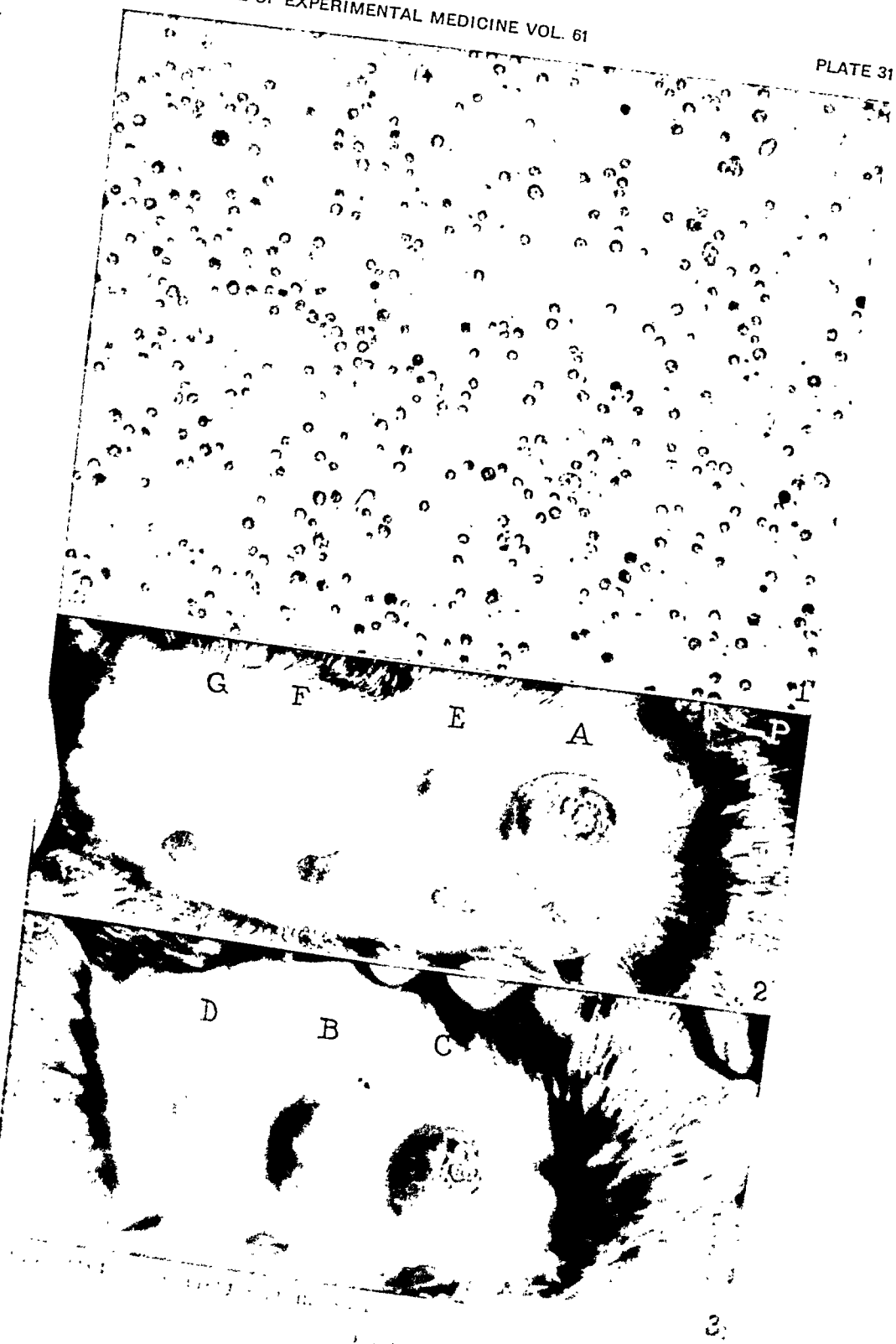
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EXPLANATION OF PLATE 31

FIG. 1. Suspension of living cells as prepared for exposure to a virus. A few minutes before the photograph was taken a drop of the suspension was placed on a slide coated with neutral red. The cells that appear dark have already segregated the dye. $\times 130$.

FIGS. 2 and 3. Shope lesions on sides of Rabbit 1 of Experiment 8 (Chart 8), photographed on the 17th day. The skin is marked with dye below the site of each injection. P = rump of the animal.

The inocula were:—A, cells washed in gel. Ty.; B, cells killed with water and washed in gel. Ty.; C, cells rotated with immune serum, killed with water and washed; D, cells killed with water, rotated with immune serum and washed; E, decelled fluid from tryptic digest; F, decelled supernatant fluid from the cultures; G, cells from the supernatant fluid. F and G gave rise late to scattered, shotty nodules, and one developed several centimeters below the main growth caused by E. $\times \frac{1}{2}$.



ENCEPHALOMYELITIS ACCOMPANIED BY MYELIN DESTRUCTION EXPERIMENTALLY PRODUCED IN MONKEYS

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PLATES 32 TO 34

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The etiology of the encephalomyelitis accompanied by demyelination that occasionally follows antirabic vaccination (1-4) and certain acute infections, *e.g.* vaccinia and measles, has been the source of many discussions, and the cause of the malady is still not definitely known. The fact that large amounts of heterologous brain material are injected into patients undergoing antirabic vaccination, has induced certain investigators (1, 5) to consider this procedure responsible in some manner for the changes found in the central nervous system of those who become paralyzed. Indeed, a few workers have been able to produce paralysis in rabbits by means of repeated injections either of heterologous brain tissue (5, 6) or of autolyzed homologous brain material (7). In spite of the fact that paralysis can be caused in rabbits in this manner, investigators (6, 7) have been unable to demonstrate lesions in the nervous system to account for its occurrence.

Rivers, Sprunt, and Berry (8) have reported that they observed an encephalomyelitis with myelin destruction in 2 of 8 monkeys that had received repeated intramuscular injections of aqueous emulsions and alcohol-ether extracts of normal rabbit brain. Inasmuch as demyelinating maladies occasionally occur spontaneously (9-15) in monkeys, these workers thought it best not to draw any definite conclusions concerning the relation of the injections to the production of the encephalomyelitis in the monkeys. The investigations have been continued, and at the present time we shall present the results of an experiment that seems to indicate that the repeated intramuscular

injections of aqueous emulsions and alcohol-ether extracts of rabbit brain into monkeys are in some way related to the occurrence of the lesions found in the central nervous system.

Methods and Materials

Monkeys.—All monkeys (*Macacus rhesus*) used in the experiment were healthy and approximately half-grown.

Fresh Aqueous Emulsions of Rabbit Brain.—The fresh aqueous emulsions of rabbit brain were prepared in the following manner: One normal rabbit brain was thoroughly ground with alundum in a mortar. 40 cc. of Locke's solution and 10 cc. of 95 per cent alcohol were added. Then the emulsion was centrifuged at speed 5 for 3 minutes. 3–5 cc. of the supernatant material were injected intramuscularly into each monkey. Fresh emulsions were made for each set of inoculations. The sterility of the materials employed was tested by means of cultures. It is fully realized that a certain percentage of the normal rabbits used may have had the spontaneous encephalitis that is found in rabbits of this country.

Alcohol-Ether Extracts of Rabbit Brain.—The brains of 4 rabbits that had been exsanguinated were removed and thoroughly ground without an abrasive in a mortar. This material was then placed in a large flask and extracted for 4 days at 37°C. with 300 cc. of 95 per cent alcohol. The alcohol was drawn off and saved. Then 300 cc. of ether were added to the brain tissue and extraction was allowed to take place at 37°C. for 6 days. The ether was removed and allowed to evaporate under the influence of heat and vacuum until only 20 cc. of a "soapy-looking" material remained. To this material were added the 300 cc. of alcohol with which the first extraction was made. The soapy-looking material went into solution. By means of heat (70°C.) and vacuum the volume of the mixed extracts was reduced to 150 cc. The concentrated alcohol-ether extract was stored in a cold room kept at 0°C. At this temperature a white waxy sediment appeared in the extract; but when the temperature of the extract was raised to 70° or 80°C. the sediment again went into solution. For the injection of each monkey 1 cc. of the alcohol-ether extract heated to 70–80°C. was added to 3 or 4 cc. of sterile distilled water. The resulting mixture consisted of a milky-looking fluid with the appearance of a Wassermann antigen.

Injections of Brain Emulsions and Extracts.—The brain emulsions and extracts were repeatedly injected intramuscularly in monkeys, the animals usually receiving 3 inoculations a week consisting either of 2 emulsions and 1 extract or of 2 extracts and 1 emulsion. The animals at times were allowed periods of rest during which no inoculations were given. An occasional animal developed an abscess at the site of inoculation and while the abscess was present the injections were discontinued. The schedule of inoculations for the group of monkeys is summarized in Table I.

Housing and Feeding of Monkeys.—2 monkeys were kept in each cage which was large enough for exercise. The cages were in a large well ventilated room.

The animals received bananas or oranges for breakfast, bread for lunch, and heated milk for supper. At times raw carrots and roasted peanuts were also included in the diet.

Control Monkeys.—8 monkeys received the repeated injections. 8 control monkeys were housed in the same room under conditions identical with those to which the test monkeys were subjected with the exception that they received no injections.

Autopsies.—Complete autopsies were performed on all 8 of the test monkeys and on 4 of the control animals. Cultures to test the sterility of the brains were made aerobically and anaerobically in meat-infusion broth, and aerobically on blood-agar, on Petroff's egg medium for acid-fast organisms, and on Sabouraud's medium for fungi.

Stains.—Sections from different parts of the cerebrum, cerebellum, pons, and cord were stained with hematoxylin and eosin, with Giemsa's stain, according to Marchi's method, and according to Kulschitzky-Wolter's modification of Weigert's myelin sheath stain.

EXPERIMENTAL

The experiment reported in this paper was planned to determine whether injections of aqueous emulsions and extracts of rabbit brain had any causal relation to the lesions described by Rivers, Sprunt, and Berry (8) in the nervous system of the monkeys used in their work. Eight monkeys (*Macacus rhesus*) received repeated intramuscular injections of aqueous emulsions and alcohol-ether extracts of normal rabbit brain made and administered according to the directions detailed above. Eight other monkeys (*Macacus rhesus*) were kept as controls in the same room under conditions identical with those to which the test animals were subjected with the exception that they received no injections. The results obtained in each animal appear below in detail under the animal's number, after which follows a description of the lesions observed in stained sections of the nervous system. It would be repetitious to describe in detail the lesions found in each monkey because the same type of pathological change occurred in every animal that became sick.

Monkey 8.—On Dec. 29, 1933, Monkey 8, having received 44 injections according to the schedule in Table I, exhibited slight ataxia, and held its head nearer the right than the left shoulder. The head could be rotated without apparent discomfort to the animal. The power in all the limbs was good. Temp. 102.4°F. On Jan. 2, 1934, the ataxia was more marked; the head was held more to the right,

and at times the chin rested on the shoulder. Temp. 102.2°F. The monkey received its 45th injection. Jan. 3, temp. 101.6°F. Jan. 4, the monkey although eating fairly well showed loss of weight. The ataxia had progressed to the extent that the animal was unable to move about without holding to the sides of the cage. Temp. 102.4°F. The animal received its 46th injection. Jan. 5, temp. 101.8°F. Jan. 6, the monkey was much weaker and more ataxic. A definite strabismus of the eyes was noted. Inasmuch as it appeared that the animal would not live much longer, it was sacrificed. While the monkey was

TABLE I

Schedule of Injections Received by Monkeys during 1933-34

Month	Day of month																														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Aug.																						w		a		w		a		w	
Sept.	a				w		a		w		a		w		a		w			a		w			a		w		a		
Oct.			w		a		w		a		w		a			w		a			w										
Nov.													a		w					a		w		a			w		a		
Dec.		w			a		w		a		w		a			w		a		w		a									
Jan.		w		a		w		a		w		a				w		a													
Feb.					w			a		w				a			w		a		w		a			w		a			
Mar.												w		a		w			a			w		a							
Apr.		w			a				w		a		w											a		w		a		w	
May		a		w										a		w		a			w		a		w						

w indicates that the monkeys received aqueous emulsion of rabbit brain.

a indicates that the monkeys received alcohol-ether extract of rabbit brain.

Eight monkeys received injections. Some became paralyzed sooner than others: 4 received 85 injections; 1 received 80; 2 received 62; 1 received 46.

under ether anesthesia and before it was killed, a cisternal puncture was made. Clear fluid with 220 cells per c.mm. (40 per cent polymorphonuclear and 60 per cent mononuclear elements) giving a doubtfully positive Pandy reaction was obtained.

Necropsy.—No evidence of tuberculosis was found. All of the organs except the lungs, which showed a slight amount of consolidation at the edges of some of the lobes, appeared approximately normal. The brain and cord exhibited no lesions to the unaided eye. Anaerobic and aerobic cultures made from different parts of the cerebrum and cerebellum in meat infusion broth, and aerobic cultures on Sabouraud's medium remained sterile. Half of the brain was fixed in Zenker-

formol solution, the other half in 10 per cent formalin. Acid-fast and Gram-Weigert stains of sections of the central nervous system showed no fungi or bacteria. Hematoxylin and eosin, Giemsa, Marchi, and modified Weigert stains showed typical lesions accompanied by demyelination, which will be described in detail later. The meninges particularly in the neighborhood of the cerebello-pontine angle were thickened and showed evidences of inflammation. Many lesions, most numerous in the neighborhood of the 4th ventricle, were found in the cerebellum and pons. In the cerebrum a slight amount of perivascular infiltration was seen in a number of sections, in one of which a large lesion also was found near the base of the occipital lobe.

Results of Animal Passages.—Each of 2 monkeys received intracerebrally 1 cc. of a 10 per cent emulsion of the brain of Monkey 8. Neither of them showed evidences of illness; one was sold 5 months later, the other is still under observation, 1 year, and is in perfect condition. Each of 2 rabbits received intracerebrally 0.25 cc. of the emulsion. Both remained well for 2 months. Then they were sacrificed, and, upon histological examination, their brains were found to be normal. Each of 6 Swiss albino mice received intracerebrally 0.03 cc. of the emulsion. All of them remained well for 2 months after which time they were discarded.

Monkey 1.—On Feb. 19, 1934, Monkey 1, that received 62 injections, showed ataxia and a right facial palsy. Temp. 101.6°F. Feb. 21, temp. 102.6°F. Feb. 23, the ataxia and right facial weakness were more pronounced. Drooling from mouth was noted for the first time. Feb. 28, the ataxia was so marked that the animal moved about with great difficulty. Mar. 1, the animal's condition was about the same. While the monkey was under ether anesthesia, a cisternal puncture was made which yielded a clear fluid with 21 cells per c.mm. (60 per cent polymorphonuclear and 40 per cent mononuclear elements) and a positive Pandy reaction. Then the animal was sacrificed.

Necropsy.—To the unaided eye the liver, spleen, kidneys, brain, and cord were negative. The lungs showed a few very small areas of consolidation. No evidence of tuberculosis was found. Cultures of the brain and cord for ordinary bacteria remained sterile. Sections of the organs other than the brain and cord showed no lesions of importance. Sections of the brain and cord showed a few typical lesions in the cerebrum, and a number in the cerebellum and pons particularly around the 4th ventricle. The only changes seen in the cord were brought out by the Marchi stain, and consisted of a degeneration of the myelin in the anterior part of the cord near the fissure and in one of the lateral bundles. Acid-fast and Gram-Weigert stains revealed no bacteria or fungi in the tissues.

Results of Animal Passages.—Each of 2 monkeys received intracerebrally 1 cc. of a 10 per cent emulsion of the brain of Monkey 1. Both remained well for 2 months after which time they were discarded. Each of 2 rabbits received intracerebrally 0.25 cc. of the brain emulsion, remained well for 2 months, and were then discarded. Each of 6 Swiss albino mice received intracerebrally 0.03 cc. of the brain emulsion. While under observation for 2 months they remained well.

Monkey 7.—On Feb. 21, 1934, Monkey 7, that received a total of 62 injections,

EXPERIMENTAL ENCEPHALOMYELITIS

showed a ptosis of both eyelids. On Feb. 28, the animal was found to be ataxic. Temp. 101.2°F. The ataxia increased gradually until Mar. 12 when the monkey was scarcely able to move about in the cage. No definite evidence of paralysis of the extremities was observed. The bilateral ptosis persisted and the animal appeared to be blind. While the monkey was under ether anesthesia a cisternal puncture was made which yielded a clear fluid containing 6 mononuclear cells per c.mm. and a slightly positive Pandy reaction. The animal was then sacrificed and all the organs were examined.

Necropsy.—To the unaided eye all the organs including the brain and cord seemed to be normal. Cultures of the brain and cord on ordinary media and on Sabouraud's medium remained sterile. Sections of the liver, spleen, kidneys, and lungs showed nothing of importance. Sections of the cerebrum, cerebellum, and pons stained with hematoxylin and eosin revealed typical lesions to be described later. Gram-Weigert and acid-fast stains showed no microorganisms. Weigert's stain showed myelin destruction, much of which was around blood vessels, in the cerebrum, cerebellum, and pons. Some degeneration of myelin in the spinal cord near the anterior fissure and in the lateral columns was made evident by means of Marchi's stain.

Results of Animal Passages.—Each of 2 rabbits received intracerebrally 0.25 cc. of an emulsion of the brain of Monkey 7. Both animals remained well for a period of 2 months and then were discarded. A guinea pig received 0.1 cc. of the brain emulsion intracerebrally, remained well for 2 months, and then was sacrificed. Stained sections of its central nervous system revealed no lesions. Each of 6 Swiss albino mice received intracerebrally 0.03 cc. of the brain emulsion, remained well for 2 months, and then was discarded.

Monkey 2.—Monkey 2 received 80 injections. On Oct. 10, 1933, a small abscess appeared in the muscles of the right leg. The injections were discontinued for a short time in order to allow the abscess and the animal was slightly ataxic. May 14, during the past 3 weeks the animal has gradually become more ataxic. No definite paralysis of the extremities was observed, but a right facial paralysis was present at this time. The injections were discontinued. May 21, the head is held turned towards the left. June 6, no appreciable change has been noted in the animal's condition since the injections were discontinued. The animal was sacrificed for examination.

Necropsy.—Aerobic and anaerobic cultures of bits of the brain and cord in meat-infusion broth, and aerobic cultures on blood-agar, on Petroff's egg medium, and on Sabouraud's medium remained sterile. To the unaided eye all the organs including the brain and cord appeared normal. Sections of the lungs, kidneys, liver, spleen, testicles, and muscle stained with hematoxylin and eosin and Giemsa's stain showed no lesions of importance. Sections of the central nervous system stained with hematoxylin and eosin and Giemsa's stain showed typical lesions in the cerebrum, cerebellum, pons, and cord. The meninges in the region of the cerebellopontine angle and at certain levels of the cord were also involved. De-

myelination in the cerebrum, cerebellum, and pons was demonstrated by means of Weigert's stain. Marchi and Weigert stains showed changes or destruction of myelin in the cord near the anterior and posterior fissures and near the surface of the cord posteriorly and laterally.

Results of Animal Passages.—One monkey received intracerebrally 1.0 cc. of an emulsion from the brain of Monkey 2. It remained well for 6 months at which time it was used in another experiment. When the animal was sacrificed at the end of the experiment all the organs were found to be essentially normal.

Monkey 3.—Monkey 3 received 85 injections. On Apr. 4, 1934, a slight ataxia was observed for the first time. May 21, the ataxia has gradually progressed. June 7, the animal has become very ataxic. No definite paralyses were observed. The animal was sacrificed for histological examination after a cisternal puncture had been made. The fluid was clear, contained 16 cells per c.mm. of which one was polymorphonuclear, and yielded a positive Pandy reaction.

Necropsy.—Cultures of bits of the brain made aerobically and anaerobically in meat infusion broth, and aerobically on blood-agar plates, on Sabouraud's medium, and on Petroff's egg medium remained sterile. All the organs including the central nervous system appeared normal to the unaided eye. Stained sections of the lungs, testicles, muscle, kidneys, pancreas, liver, adrenals, and spleen showed no lesions of importance. Sections of brain and cord stained with hematoxylin and eosin, Giemsa's stain, modified Weigert's stain, and Marchi's stain showed typical extensive lesions in the cerebellum and pons. The meninges in the region of the cerebellopontine angle were involved. The cerebrum and cord showed few if any noteworthy changes.

Results of Animal Passages.—One monkey received intracerebrally 1.0 cc. of an emulsion of bits of the brain of Monkey 3 and has remained well for a period of 7 months. The animal is still under observation.

Monkey 6.—Monkey 6 received 85 injections. On Oct. 10, 1933, an abscess occurred in the subcutaneous tissues of the right leg, and the injections were discontinued for 2 weeks in order to allow it to heal. On May 21, 1934, the animal was slightly unsteady in its movements. May 31, the monkey was definitely ataxic. June 6, the ataxia has progressed very rapidly and the animal has great difficulty in getting around in the cage. No definite paralyses were noted. The head was held twisted to the left. While the monkey was under ether anesthesia a cisternal puncture was made which yielded a clear fluid in which numerous small flocculi were seen. The fluid contained 280 cells per c.mm. (18 per cent polymorphonuclear and 82 per cent mononuclear elements) and gave a positive Pandy reaction. The animal was sacrificed for histological examination.

Necropsy.—Cultures of the brain made aerobically and anaerobically in meat infusion broth, and aerobically on blood-agar, on Sabouraud's medium, and Petroff's medium remained sterile. All the organs appeared normal to the unaided eye. Stained sections of the lungs, liver, spleen, pancreas, adrenals, testicles, kidneys, and muscle showed no significant changes. Sections of the brain and cord stained with hematoxylin and eosin and Giemsa's stain revealed typical

showed a ptosis of both eyelids. On Feb. 28, the animal was found to be ataxic. Temp. 101.2°F. The ataxia increased gradually until Mar. 12 when the monkey was scarcely able to move about in the cage. No definite evidence of paralysis of the extremities was observed. The bilateral ptosis persisted and the animal appeared to be blind. While the monkey was under ether anesthesia a cisternal puncture was made which yielded a clear fluid containing 6 mononuclear cells per c.mm. and a slightly positive Pandey reaction. The animal was then sacrificed and all the organs were examined.

Necropsy.—To the unaided eye all the organs including the brain and cord seemed to be normal. Cultures of the brain and cord on ordinary media and on Sabouraud's medium remained sterile. Sections of the liver, spleen, kidneys, and lungs showed nothing of importance. Sections of the cerebrum, cerebellum, and pons stained with hematoxylin and eosin revealed typical lesions to be described later. Gram-Weigert and acid-fast stains showed no microorganisms. Weigert's stain showed myelin destruction, much of which was around blood vessels, in the cerebrum, cerebellum, and pons. Some degeneration of myelin in the spinal cord near the anterior fissure and in the lateral columns was made evident by means of Marchi's stain.

Results of Animal Passages.—Each of 2 rabbits received intracerebrally 0.25 cc. of an emulsion of the brain of Monkey 7. Both animals remained well for a period of 2 months and then were discarded. A guinea pig received 0.1 cc. of the brain emulsion intracerebrally, remained well for 2 months, and then was sacrificed. Stained sections of its central nervous system revealed no lesions. Each of 6 Swiss albino mice received intracerebrally 0.03 cc. of the brain emulsion, remained well for 2 months, and then was discarded.

Monkey 2.—Monkey 2 received 80 injections. On Oct. 10, 1933, a small abscess appeared in the muscles of the right leg. The injections were discontinued for a short time in order to allow the abscess to heal. On Apr. 24, 1934, the face of the animal had a mask-like appearance and the animal was slightly ataxic. May 14, during the past 3 weeks the animal has gradually become more ataxic. No definite paralysis of the extremities was observed, but a right facial paralysis was present at this time. The injections were discontinued. May 21, the head is held turned towards the left. June 6, no appreciable change has been noted in the animal's condition since the injections were discontinued. The animal was sacrificed for examination.

Necropsy.—Aerobic and anaerobic cultures of bits of the brain and cord in meat-infusion broth, and aerobic cultures on blood-agar, on Petroff's egg medium, and on Sabouraud's medium remained sterile. To the unaided eye all the organs including the brain and cord appeared normal. Sections of the lungs, kidneys, liver, spleen, testicles, and muscle stained with hematoxylin and eosin and Giemsa's stain showed no lesions of importance. Sections of the central nervous system stained with hematoxylin and eosin and Giemsa's stain showed typical lesions in the cerebrum, cerebellum, pons, and cord. The meninges in the region of the cerebellopontine angle and at certain levels of the cord were also involved. De-

myelination in the cerebrum, cerebellum, and pons was demonstrated by means of Weigert's stain. Marchi and Weigert stains showed changes or destruction of myelin in the cord near the anterior and posterior fissures and near the surface of the cord posteriorly and laterally.

Results of Animal Passages.—One monkey received intracerebrally 1.0 cc. of an emulsion from the brain of Monkey 2. It remained well for 6 months at which time it was used in another experiment. When the animal was sacrificed at the end of the experiment all the organs were found to be essentially normal.

Monkey 3.—Monkey 3 received 85 injections. On Apr. 4, 1934, a slight ataxia was observed for the first time. May 21, the ataxia has gradually progressed. The animal has become very ataxic. No definite paralyzes were observed. The animal was sacrificed for histological examination after a cisternal puncture had been made. The fluid was clear, contained 16 cells per c.mm. of which one was polymorphonuclear, and yielded a positive Pandy reaction.

Necropsy.—Cultures of bits of the brain made aerobically and anaerobically in meat infusion broth, and on Petroff's egg medium remained sterile. All the organs including the central nervous system appeared normal to the unaided eye. Stained sections of the lungs, testicles, muscle, kidneys, pancreas, liver, adrenals, and spleen showed no lesions of importance. Sections of brain and cord stained with hematoxylin and eosin, Giemsa's stain, modified Weigert's stain, and Marchi's stain showed typical extensive lesions in the cerebellum and pons. The meninges in the region of the cerebellopontine angle were involved. The cerebrum and cord showed few if any noteworthy changes.

Results of Animal Passages.—One monkey received intracerebrally 1.0 cc. of an emulsion of bits of the brain of Monkey 3 and has remained well for a period of 7 months. The animal is still under observation.

Monkey 6.—Monkey 6 received 85 injections. On Oct. 10, 1933, an abscess occurred in the subcutaneous tissues of the right leg, and the injections were discontinued for 2 weeks in order to allow it to heal. On May 21, 1934, the animal was slightly unsteady in its movements. May 31, the monkey was definitely ataxic. June 6, the ataxia has progressed very rapidly and the animal has great difficulty in getting around in the cage. No definite paralyzes were noted. The head was held twisted to the left. While the monkey was under ether anesthesia a cisternal puncture was made which yielded a clear fluid in which numerous small flocculi were seen. The fluid contained 280 cells per c.mm. (18 per cent polymorphonuclear and 82 per cent mononuclear elements) and gave a positive Pandy reaction. The animal was sacrificed for histological examination.

Necropsy.—Cultures of the brain made aerobically and anaerobically in meat infusion broth, and aerobically on blood-agar, on Sabouraud's medium, and Petroff's medium remained sterile. All the organs appeared normal to the unaided eye. Stained sections of the lungs, liver, spleen, pancreas, adrenals, testicles, kidneys, and muscle showed no significant changes. Sections of the brain and cord stained with hematoxylin and eosin and Giemsa's stain revealed typical

lesions in the cerebrum near the lateral ventricles, in the cerebellum and pons in the neighborhood of the 4th ventricle and cerebellopontine angle. The meninges in this neighborhood were also involved. Weigert's stain demonstrated destruction of myelin much of which had occurred around blood vessels. Marchi's stain showed degenerating myelin in the cord near the anterior fissure and in the lateral columns near the surface.

Results of Animal Passages.—A monkey received intracerebrally 1.0 cc. of an emulsion of bits of the brain of Monkey 6. The animal has remained well for a period of 7 months and is still under observation.

Monkey 4.—Monkey 4 received 85 injections. The animal never showed definite signs of involvement of the central nervous system. Nevertheless, during the last 3 months of the experiment it was docile and refused to run around very much. On June 7, 1934, while the animal was under ether anesthesia a cisternal puncture was made which yielded a clear fluid containing 42 cells per c.mm. (54 per cent polymorphonuclear and 46 per cent mononuclear elements) and gave a negative Pandy reaction. The monkey was then sacrificed for histological examination.

Necropsy.—Aerobic and anaerobic cultures of bits of the brain in meat infusion broth, and aerobic cultures on blood-agar, on Sabouraud's medium, and on Petroff's medium remained sterile. All the organs including the brain and cord appeared normal to the unaided eye. Stained sections of the lungs, kidneys, spleen, pancreas, adrenals, liver, testicles, and muscle showed no lesions of significance. Section of the brain and cord stained with hematoxylin and eosin, and modified Weigert's stain revealed a few typical lesions with destruction of myelin in the cerebrum, near the ventricles, and in the cerebellum and pons. Marchi's stain showed a few degenerating myelin sheaths near the anterior fissure of the cord.

Monkey 5.—Monkey 5 received 85 injections. At no time during the experiment did the animal exhibit signs of involvement of the central nervous system, and on June 6, 1934, it was sacrificed for histological examination after a cisternal puncture had been made. The fluid obtained was clear, contained 8 mononuclear cells per c.mm., and yielded a negative Pandy reaction.

Necropsy.—All cultures of the brain remained sterile. All of the organs including the brain and cord appeared normal to the unaided eye, and stained sections of them showed no significant lesions.

Control Monkeys.—Monkeys 9-16 were kept under conditions identical with those to which Monkeys 1-8 were subjected except that they received no injections. At no time did any of the control animals exhibit signs of involvement of the central nervous system. When the experiment was terminated 4 of the control animals, Monkeys 9-12, were sacrificed for complete histological examination. None of the organs including the brain and cord revealed lesions of significance.

The results of the experiment detailed above are summarized in Table II, an examination of which reveals that 6 of the 8 monkeys that received repeated intramuscular injections of aqueous emulsions

and alcohol-ether extracts of normal rabbit brain tissue developed signs of involvement of the central nervous system and that in the brains of 7 of the 8 animals lesions, extensive in many instances, were found by means of histological examinations. None of the control monkeys became sick and in the brains of 4 of them no lesions were found when histological examinations were made. The other 4 control monkeys were not sacrificed.

Ptoxis of the eyelids, mask-like expression of the face, facial paralysis, abnormal position of the head (held to the right or to the left), blindness, and ataxia were the usual clinical signs and symptoms noted. Little or no paralysis of the extremities was detected. The animals did not have fever. There was a pleocytosis and an increased amount of globulin in the majority of the spinal fluids of the sick monkeys.

The injections were continued even after the monkeys evidenced signs of involvement of the central nervous system. This was done in order to obtain pronounced lesions for histological investigations. In one instance (Monkey 2), however, the injections were discontinued after the ataxia had developed. The animal was then observed for 3 weeks during which time no appreciable change in its condition was noted. At least there was no evidence that the animal would recover.

The localization of the lesions in the central nervous system is interesting. The cerebellum and pons, particularly in the neighborhood of the 4th ventricle, seemed to be the portions of the brain most frequently and most severely attacked. The cerebrum exhibited lesions which appeared most often near the ventricles. Nevertheless, some lesions were seen in the cortex. Except for tract degenerations which were seen in many instances, the cord of only 1 monkey showed pathological changes. The meninges, especially in the vicinity of the cerebellopontine angle, were usually, but not always, involved.

Description of the Pathological Changes in the Central Nervous System:

Inasmuch as the pathological changes in the brains and cords of the different monkeys were almost identical in character, a general description of them will be made instead of giving in detail the findings in individual animals.

For the most part, the pathological changes seemed to have some

relation to the blood vessels. This was particularly true of the small lesions. When the changes became extensive, however, the perivascular distribution was not always easily discernible. Alterations in the vessels themselves, such as thrombosis, were not found. Both the gray and white matter were involved, the latter being more severely implicated.

The first stage in the development of the lesions was apparently a proliferation of cells giving rise to elements larger than those usually found in the supporting tissues of the brain (Figs. 1, 6, 7). Perivascular changes (Figs. 1, 5) made their appearance early and consisted of proliferation of glial elements and an infiltration of mononuclear cells and polymorphonuclear eosinophiles. As the pathological changes progressed compound granular corpuscles became exceedingly numerous, and in some of the large lesions hemorrhages occurred (Figs. 3, 4). One of the most striking features of the pathological picture was the presence of large numbers of polymorphonuclear eosinophiles. In many sections indefinite crystal-like structures were seen some of which were intracellularly situated.

Foreign body giant cells (Figs. 1, 2, 6) containing ingested cells and debris were not infrequently found in early as well as in advanced lesions. In addition to the giant cells there were multinucleated elements (Fig. 2) somewhat similar to the "globoid" cells seen in sections of the brain of a case of Schilder's disease and described by Collier & Greenfield (16) in the following manner:

"In all regions where myelin destruction was active, particularly in those regions where it appeared to be of more recent date, there were large 'globoid' cells of peculiar character. Their nuclei were always multiple and sometimes were numerous and were arranged as a chain of thin flattened nuclei under the capsule of the cell.

By means of a modified Weigert stain myelin destruction was noted in all parts of the brain. It was most extensive, however, around the ventricles and in the pons and cerebellum (Figs. 9, 10). Much of the demyelination was perivascularly situated (Figs. 11, 12). Often bits of the broken myelin sheaths had the appearance of large bacteria (Fig. 12). At other times they appeared as globules within phagocytic cells (Fig. 13).

DISCUSSION

The results of the experiment reported at this time are similar to those described by Rivers, Sprunt, and Berry (8). The pathological changes are identical in both sets of animals and in some respects do not resemble the spontaneous lesions of monkeys that have been described on previous occasions by other workers (9-15).

The appearance of foreign body giant cells in the brains of the affected animals is extremely interesting, and the character of the lesions observed is such that one would suspect that an infectious agent caused them. Nevertheless, we have been unable to demonstrate the presence of such an agent by means of stains and cultures. Furthermore, inoculation of monkeys, rabbits, guinea pigs, and mice with emulsions of the brains involved failed to disclose a transmissible agent. However, failure on our part to find an infectious agent does not necessarily mean that one was not present.

The fact that the control animals remained well seems to indicate clearly that the pathological changes which occurred in the brains of the treated monkeys were in some manner, either directly or indirectly, brought about as a result of the repeated intramuscular injections of aqueous emulsions and alcohol-ether extracts of sterile normal rabbit brain. Whether the aqueous emulsions or the extracts were responsible remains to be determined. Furthermore, it is possible that some obscure infectious agent was activated by the injections. If that be the case, then 7 of the 8 monkeys carried the agent and at least 2 of the 8 monkeys treated by Rivers, Sprunt, and Berry (8) also carried it.

The relation of our results to the paralysis accompanied by destruction of myelin that is known to follow the repeated injections of emulsified rabbit brain containing fixed rabic virus used in the vaccination of human beings against rabies, is not clear. In the Pasteur treatment of human beings 14 to 21 injections are made, and only about 1 of every 4000 vaccinated individuals becomes paralyzed. Each of our animals received more injections than are used in the Pasteur treatment, the smallest number being 46, the largest 85. It was not possible to buy and house several thousand monkeys. Consequently, we decided to use a large number of injections in a small group of animals. In any event we have experimentally produced lesions accompanied by myelin destruction in the brain of monkeys.

Further work is under way to determine the nature and the mechanism of the production of such lesions.

SUMMARY

The repeated intramuscular injections of aqueous emulsions and alcohol-ether extracts of sterile normal rabbit brains in some manner produced pathological changes accompanied by myelin destruction in the brains of 7 of 8 monkeys (*Macacus rhesus*). Eight control monkeys remained well. Cultures from the involved brains remained sterile, and no transmissible agent was demonstrated by means of intracerebral inoculations of emulsions of bits of the brains into monkeys, rabbits, guinea pigs, and white mice.

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EXPLANATION OF PLATES

PLATE 32

- FIG. 1. Perivascular infiltration and giant cells. Hematoxylin and eosin. $\times 100$.
- FIG. 2. Two definite foreign body giant cells and a cell somewhat similar to the "globoid" cell described by Collier and Greenfield. Hematoxylin and eosin. $\times 400$.
- FIGS. 3 and 4. Typical advanced lesions. In Fig. 4 a hemorrhage has occurred in one of the lesions. Hematoxylin and eosin. $\times 100$.

PLATE 33

FIG. 5. Type of perivascular infiltration observed; proliferation of glial elements, infiltration of mononuclear elements and polymorphonuclear eosinophiles. Hematoxylin and eosin. $\times 400$.

FIGS. 6 and 7. Early lesions showing proliferative changes without the presence of polymorphonuclear eosinophiles. Hematoxylin and eosin. $\times 400$.

FIG. 8. Edge of an advanced lesion showing proliferative changes and infiltration of polymorphonuclear elements. Hematoxylin and eosin. $\times 400$.

PLATE 34

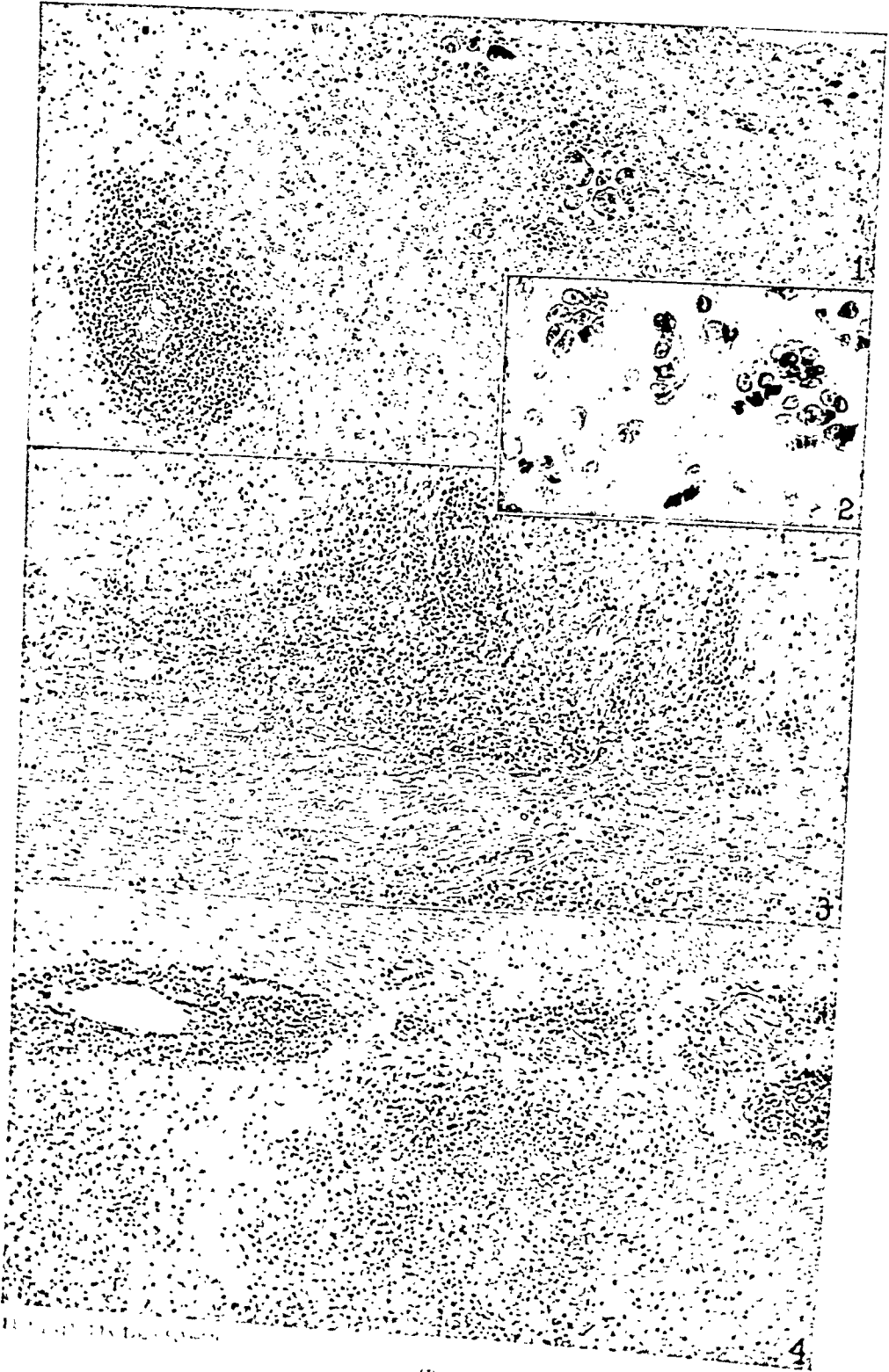
FIG. 9. Marked destruction of myelin in the pons, particularly around the central canal. Modification of Weigert's stain. $\times 3.5$.

FIG. 10. Extensive destruction of myelin in the pons and cerebellum. Modification of Weigert's stain. $\times 3.5$.

FIG. 11. Photograph to illustrate the perivascular distribution of the myelin destruction. Modification of Weigert's stain. $\times 37$.

FIG. 12. Perivascular demyelination in the cerebral cortex. Note the broken myelin sheaths, bits of which have the appearance of large bacteria. Modification of Weigert's stain. $\times 400$.

FIG. 13. Phagocytic cells filled with globules of myelin. Modification of Weigert's stain. $\times 400$.



(Haver and S. 1930. *Experimental encephalomyelitis*)

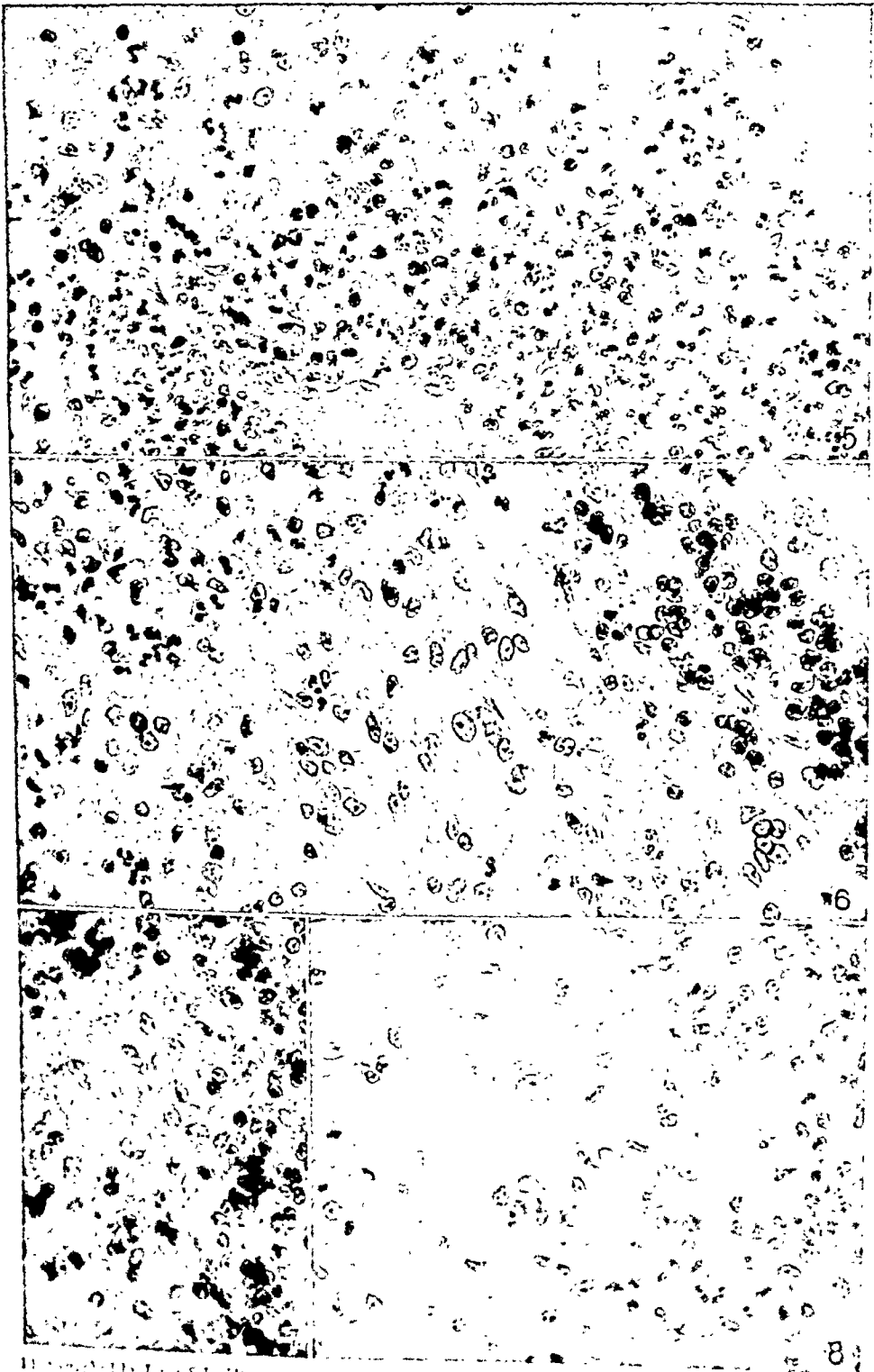


Fig. 1. (A) H. L. S. 1000



8



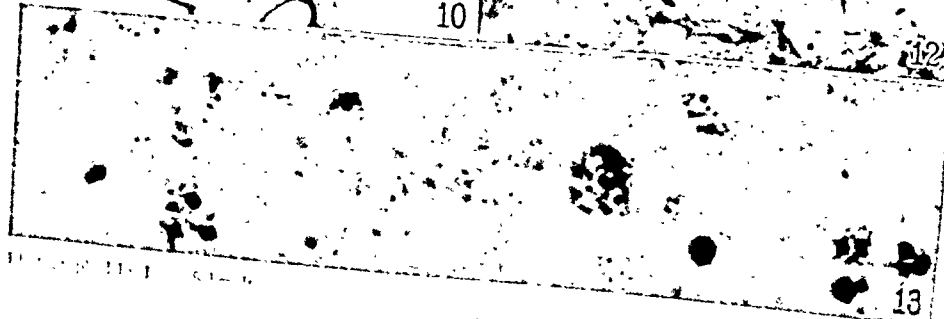
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10



11



12

13

(Hirsch and Schwentker: Experimental encephalomyelitis)



THE RELATION BETWEEN THE TYPE SPECIFIC CARBOHYDRATES OF PNEUMOCOCCI AND THE BLOOD GROUP SPECIFIC SUBSTANCE A

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The correlation between Forssman's antigen and the blood group specific substance of human blood group A is well established (1). The content of either one of these antigens in a number of bacteria has been studied by several authors. Forssman's antigen was found in various strains of *B. shigae*, *B. paratyphosus*, *B. leptosepticus*, etc. It also occurs in pneumococci (2, 3). The relationship between pneumococci and the blood group specific substances A and B has been the subject of recent investigations by Baily and Shorb (4). These authors observed agglutination of human blood cells by various antipneumococcic sera irrespective of type. Concerning the carbohydrate nature of the blood group substance A, we refer to Landsteiner (5), and Brahn, Schiff, and Weinmann (6). Freudenberg and Eichel (7) succeeded in isolating from urine of men belonging to group A a carbohydrate which in its chemical structure is closely related to the type specific carbohydrate of pneumococci prepared by Avery, Heidelberger, and Goebel.

The following study deals with the relationship between the soluble specific substances of pneumococci and the blood group specific substance A. In a study of this kind, one has to take into account the widespread occurrence in animals of Forssman's antigen and blood group antigens as well as of the corresponding antibodies.

Methods

There are various methods by which to demonstrate the presence of blood group specific substances in cells, tissues, organs, and juices; first, the specific inhibition of the iso-agglutination of human blood cells; second, the complement

TABLE I
Hemolysis of Sheep Cells by Complement and A Antiserum Mixed with Soluble Specific Pneumococcus Carbohydrate (Hemolysis Inhibition Test)

Amount of 1 per cent solution of SSS (or peptone) in 0.4 cc. of mixture with antiserum cc.	Degree of sheep cell hemolysis by mixture of A antiserum with			
	SSS I	SSS II	SSS III	Peptone
0.2	c.	0	0	0
0.1	c.	tr.	0	0
0.05	c.	c.	0	0
0.025	c.	c.	0	0
0.0125	c.	c.	tr.	0
0.0062	c.	c.	m.	tr.
0	c.	c.	c.	c.

c. = complete hemolysis.
 ac. = almost complete hemolysis.
 m. = moderate hemolysis.
 tr. = traces of hemolysis.
 0 = no hemolysis.

TABLE II
Hemolysis of Sheep Cells by Complement and A Antiserum Treated with Soluble Specific Pneumococcus Substances

Amount of 1 per cent solution of SSS in 0.4 cc. mixture with antiserum cc.	Degree of hemolysis by mixture of A antiserum with		
	SSS I acetylated	SSS I de-acetylated	SSS III
0.1	0	m.	0
0.05	0	ac.	0
0.025	0	c.	0
0.0125	0	c.	tr.
0.0062	0	c.	m.
0.0031	0	c.	ac.
0.0015	0	c.	c.
0.00078	0	c.	c.
0.00039	0	c.	c.
0.00019	0	c.	c.
0.000095	tr.	c.	c.
0	tr.	c.	c.
	c.	c.	c.

For explanation of abbreviations see Table I.
 drate, recently isolated by Avery and Goebel (8) as the genuine type specific antigen. The test was set up as follows:

Decreasing amounts of acetyl polysaccharide, of de-acetylated polysaccharide of Type I, and also of soluble specific substance, Type III, made up to 0.2 cc., were mixed with 0.2 cc. of a suitable dilution of a group specific A-antiserum (rabbit) and kept for 20 minutes at room temperature. To these were added 0.2 cc. of a 5 per cent suspension of sheep cells and 0.2 cc. guinea pig serum in a dilution of 1:20. Hemolysis was recorded after 60 minutes incubation at 37°C.

There is a striking difference between the acetyl and the de-acetylated polysaccharides, Type I. The acetyl polysaccharide inhibits sheep cell hemolysis by group specific A-antiserum to a high degree, while the de-acetylated product is almost completely ineffective. The inhibitory potency of the acetyl polysaccharide, Type I, towards the group specific sheep cell hemolysis exceeds that of the soluble specific substance, Type III.

*Complement Fixation Test of Group Specific A-Antiserum and
Pneumococcus Carbohydrates*

Experiment 3.—The question arose whether similar results could be obtained with the complement fixation test. To this end, an experiment was set up in the following manner.

Decreasing amounts (total volume 0.2 cc.) of acetyl polysaccharide, Type I, of de-acetylated polysaccharide of the same type, of polysaccharide, Type III, and of peptone (Witte) were treated for 1 hour at 37°C. with 0.2 cc. of a group specific A-antiserum (dilution 1:10), and 0.2 cc. of guinea pig serum (1:20). To these were added 0.4 cc. of an equal mixture of 5 per cent sheep cell suspension and a suitably diluted sheep cell antiserum (rabbit). The resulting hemolysis was noted (α) after 15 minutes, and (β) after 30 minutes incubation at 37°C.

Table III shows that the acetyl carbohydrate caused a marked complement fixation with the group specific A-antiserum, while the de-acetylated derivative was completely negative in this respect. The soluble specific substance, Type III, showed only a slight positive reaction.

*Inhibition of Iso-agglutination of Human Group A Corpuscles by
Pneumococcus Polysaccharides*

Experiment 4.—The inhibitory influence of the soluble specific substance of pneumococci upon the iso-agglutination of human red blood

TABLE III

Hemolysis of Sheep Cells by Sheep Cell Antiserum and Complement after Treatment of the Latter with A Antiserum and Soluble Specific Substances of Pneumococci (Complement Fixation Test)

Amount of 1 per cent solution of SSS (or peptone) in 0.6 cc. mixture with A antiserum and complement	Degree of hemolysis							
	SSS I acetylated		SSS I de-acetylated		SSS III		Peptone	
	α	β	α	β	α	β	α	β
0.05	0	m.	c.	β	α	β	α	β
0.025	0	0	c.	c.	0	c.	0	0
0.0125	0	0	c.	c.	tr.	c.	0	0
0.0062	0	0	c.	c.	c.	c.	0	m.
0.0031	0	0	c.	c.	c.	c.	0	c.
0.0015	0	0	c.	c.	c.	c.	0	c.
0.00078	0	tr.	c.	c.	c.	c.	tr.	c.
0.00039	0	ac.	c.	c.	c.	c.	m.	c.
0	0	c.	c.	c.	c.	c.	c.	c.
	c.	c.	c.	c.	c.	c.	c.	c.

α = hemolysis after 15 minutes.
 β = hemolysis after 30 minutes.

Other abbreviations as in preceding tables.

TABLE IV

Agglutination of Human Red Blood Cells of Group A by Serum Group O after Treatment of the Latter with Soluble Specific Substance

Amount of 1 per cent solution of SSS in 0.2 cc. mixture with human O serum	Iso-hemagglutination by human O serum treated with					
	SSS I acetylated		SSS I de-acetylated		SSS III	
	α	β	α	β	α	β
0.05	—	—	++	++++	++	++++
0.0167	—	+	++	++++	++	++++
0.0056	—	++	++	++++	++	++++
0.0018	—	++	++	++++	++	++++
0.0006	±	+++	++	++++	++	++++
0	++	++++	++	++++	++	++++

— = no agglutination.

± = slight agglutination.

++ = marked agglutination.

+++ = strong agglutination.

++++ = very strong agglutination.

α = after 1 hour.

β = after 10 hours.

cells by normal human serum containing the corresponding iso-agglutinins was demonstrated as follows:

Decreasing amounts (total volume 0.1 cc.) of acetyl and de-acetylated polysaccharide, Type I, and of polysaccharide, Type III, were incubated for 20 minutes at room temperature with 0.1 cc. of inactivated human serum belonging to group O (dilution 1:1). To this was added 0.1 cc. of a 1 per cent suspension of human blood cells belonging to group A. Agglutination was noted (α) after 1 hour, and (β) after 10 hours incubation at room temperature.

The acetyl polysaccharide, Type I, displayed a marked inhibitory influence on the group specific iso-agglutination of human blood cells of group A, in contrast to the de-acetylated compound of the same type. However, in comparison with the inhibition of sheep cell hemolysis by a group specific A-antiserum, much larger amounts of the acetyl polysaccharide were necessary. It may be remarked that the iso-agglutination of blood cells of group B was not influenced at all in a parallel experiment.

Schiff, Akune, and Weiler (9, 10) have described an agent in the feces and in the saliva which destroys the group specific substances. These authors regard the "blood group enzyme" as a product of the body itself, while Witebsky and Satoh (12, 13) and recently Sievers (14) are of the opinion that this agent augments itself and is not a secretion product of the organism. The question as to the nature of this blood group-destroying agent need not be discussed here. The one fact may be mentioned that one can obtain sterile Berkefeld filtrates of feces (12) which also destroy the blood group specific substances.

In view of the above described relationship between the soluble specific substances of pneumococci and the blood group substances of man, we investigated the effects of such a Berkefeld filtrate of feces on the soluble specific pneumococcus substances.

Effect of Blood Group Enzyme on Acetyl Polysaccharide, Type I, as Demonstrated by Hemolysis Inhibition Test

Experiment 5.—0.5 cc. of a $\frac{1}{3}$ per cent solution of acetyl polysaccharide, Type I, was mixed with 0.5 cc. of an effective feces filtrate, and with 0.5 cc. of the same filtrate, inactivated by heating for 20 minutes at 60°C. These mixtures were kept at 37°C. overnight. Then, the hemolysis inhibition test was set up as

in Experiment 1. The effectiveness of feces filtrates was proved in this and similar experiments by its ability to destroy the blood group substances.

Table V shows that the acetyl polysaccharide lost its potency to inhibit sheep cell hemolysis by a group specific A-antiserum after being digested with effective feces filtrate, while it still inhibited the sheep cell hemolysis when digested with feces filtrate, inactivated by heat. The loss of reactivity toward the group specific A-antiserum of

TABLE V
Hemolysis of Sheep Cells with Complement and A Antiserum Treated with Acetylated SSS Type I Which Had Been Digested Previously with Feces Filtrate

Amount of 1/6 per cent solution of SSS in 0.4 cc. of mixture with antiserum cc.	Degree of hemolysis in hemolysis inhibition test with acetylated SSS I digested with			
	Effective		Ineffective	
	α	β	α	β
0.2	tr.	c.	0	0
0.1	m.	c.	0	0
0.05	m.	c.	0	0
0.025	m.	c.	0	0
0.0125	ac.	c.	0	0
0.0062	ac.	c.	0	0
0.0031	ac.	c.	0	0
0.0015	ac.	c.	0	0
0.00078	ac.	c.	0	c.
0.00039	ac.	c.	m.	c.
0.00019	c.	c.	m.	c.
0.00009	c.	c.	ac.	c.
			ac.	c.

Abbreviations as in Table I.

α = after 20 minutes.

β = after 40 minutes.

the acetyl carbohydrate by digestion with feces filtrate could also be demonstrated by the complement fixation test.

It may be mentioned in this connection, that the soluble specific substance of pneumococci, Type III, also loses its reactivity toward the group specific A-antiserum by digestion with effective feces filtrate.

Inhibition of Iso-agglutination by Acetyl Polysaccharide Abolished by Blood Group Enzyme

Experiment 6.—The following observation confirms the destructive influence of active feces filtrate on the acetyl carbohydrate, Type I.

Equal amounts of 0.5 cc. of 0.2 per cent solution of the acetyl substance were mixed with 0.5 cc. effective and 0.5 cc. inactivated feces filtrate, and kept at 37°C. overnight. Decreasing amounts of these mixtures, made up to 0.1 cc., were then digested for 20 minutes at room temperature with 0.1 cc. of an inactivated human serum belonging to blood group O (solution 1:1). After this, 0.1 cc. of a 1 per cent suspension of human blood cells, group A, was added. The resulting agglutination was recorded (α) after 20 minutes and (β) after 50 minutes incubation at room temperature.

As can be seen from Table VI, the acetyl polysaccharide, after having been digested with active feces filtrate, has lost its potency to

TABLE VI

Agglutination of Human Blood Cells of Group A by Serum of Group O Treated with Acetylated Type I Polysaccharide Which Had Been Digested Previously with Feces Filtrate

Amount of digested 1/10 per cent acetyl SSS I solution in 0.2 cc. mixture with group O serum	Degree of iso-hemagglutination by human O serum mixed with acetylated SSS Type I, digested with			
	Active		Inactivated	
cc.	α	β	α	β
0.1	—	—	—	++
0.05	—	—	+	++++
0.025	—	—	+	++++
0.012	—	±	+	++++
0.006	—	+	++	++++
0	+++	++++	+++	++++

Symbols as in Table IV.

α = agglutination after 20 minutes.

β = agglutination after 50 minutes.

inhibit the agglutination of human blood cells of group A by a serum of group O as in Experiment 4.

Effect of Blood Group Enzyme on the Precipitability of Acetyl Substance by Pneumococcus Type I Antiserum, Previously Absorbed with De-acetylated Polysaccharide

Experiment 7.—A Pneumococcus Type I antiserum, previously absorbed with de-acetylated polysaccharide, still precipitates the acetyl substance according to Avery and Goebel (8). We mixed, therefore, 10 cc. of a 1:1 diluted Pneumococcus Type I antiserum (horse) with 0.5 cc. of a 0.1 per cent solution of the de-

acetylated compound and kept it for 2 hours at 37°C. and then overnight in the ice chest. The resulting heavy precipitate was centrifuged, and the entire procedure was repeated twice with the supernatant fluid. The final supernatant fluid was called "absorbed Type I antiserum." The test was set up as follows: Acetyl carbohydrate was digested with effective and with ineffective feces filtrate at 37°C. overnight. Then, these specimens were tested against the absorbed Type I antiserum simultaneously with acetyl polysaccharide and with de-acetylated polysaccharide as controls. Decreasing amounts of these four specimens (total volume 0.1 cc.) were mixed with 0.3 cc. of the absorbed Type I antiserum and

TABLE VII
Precipitation of Soluble Specific Substance of Type I Pneumococcus with Absorbed Pneumococcus Type I Antiserum

Specific Substance of Type I Pneumococcus with Absorbed Pneumococcus Type I Antiserum									
Amount of 1/10 per cent solution of soluble specific substances in 0.4 cc. total volume	Degree of precipitation of Pneumococcus Type I antiserum, previously treated with de-acetylated SSS Type I, when mixed with SSS Type I								
	Acetylated						De-acetylated		
	Digested with								
	Active		Inactivated		Control				
	Feces	filtrate							
cc.	α	β	α	β	α	β	α	β	
0.1	—	++	—	+	—	+	—	—	
0.03	++	++++	++	++++	+	+++	—	—	
0.01	+++	++++	+++	++++	+	+++	—	—	
0.003	++	+++	++	+++	+	+++	—	—	
0.001	+	++	+	++	+	++	—	—	
0	—	—	—	—	—	—	—	—	

For symbols compare Table IV.
 α = precipitation after 10 minutes.
 β = precipitation after 1 hour.

For symbols compare Table IV.

α = precipitation after 10 minutes.

β = precipitation after 4 hours.

kept at 37°C. for 2 hours and subsequently for the same period at ice box temperature. The resulting precipitation (α) after 10 minutes and (β) after 4 hours is recorded in Table VII.

The absorbed Type I antiserum still reacted with the acetyl polysaccharide, but not with the de-acetylated product, thus confirming the statements of Avery and Goebel. However, there was no difference between the acetyl polysaccharide treated with effective and with inactivated feces filtrate respectively (nor with the untreated control). The reactivity of all three specimens toward the absorbed Type I antiserum was almost identical, in spite of the fact that the first

specimen had almost completely lost its reactivity against the group specific A-antiserum.

DISCUSSION

In a comparison of the specific carbohydrates of the three main types of pneumococcus, the acetyl polysaccharide of Type I is highest in reactivity towards antisera obtained from rabbits by immunization against human blood corpuscles of group A. The close relationship between this bacterial antigen and the iso-agglutinin A can be demonstrated by a number of methods. The acetyl substance exerts an inhibitory influence on sheep cell hemolysis by group A-antiserum up to a dilution of about 1:1,000,000 of its 1 per cent solution when especially titrated with optimal dilutions of complement and antiserum. Thus, the bacterial product proves to be almost as active toward the A-antiserum as the carbohydrate of Freudenberg and Eichel which acts in amounts of 1/100 to 1/200 γ . Like Freudenberg and Eichel's carbohydrate, isolated from urine of human blood group A subjects, it inhibits the corresponding iso-agglutination. However, in this type of test the necessary amount of the pneumococcus polysaccharide was found to be much higher than in the hemolysis inhibition test, in agreement with observations made by Jorpes on Freudenberg and Eichel's carbohydrate (7).

There is still another method suitable to prove the existence of A-antigen in cells and tissues. Active immunization of rabbits with material containing this antigen, may result in the production of group specific A-antibodies. Only a certain percentage of rabbits, namely the individuals lacking the A-antigen in their organs, are able to produce A-antibodies. In these, one may expect that immunization against *Pneumococcus* Type I could simultaneously produce antibodies against the A-like antigenicity, observed by us in the genuine carbohydrate of this type. Only small amounts of six individual *Pneumococcus* Type I antisera (rabbit) were at our disposal, two of which contained group specific A-antibodies. However, since the normal A-antibody in serum of rabbits, lacking A-antigen in their body, would increase also upon any non-specific antigenic irritation, our observations do not permit the assumption that the moderate amounts of group specific A-antibodies present are due to a specific

immunizing effect. Further experiments will be necessary in order to elucidate this point (*cf.* 4).

Of the other type specific carbohydrates, that of Type III was approximately 100 times less effective than the acetyl derivative of Type I in the hemolysis inhibition test, and about 20 times less effective as concerns complement fixation. In the iso-agglutination inhibition test no action by polysaccharide, Type III, could be detected at all. Type II, in turn, was much weaker than Type III in the hemolysis inhibition test. Unless the cross-relationship between the antigen of *Pneumococcus* Type I and human blood group A is to be considered a fortuitous one, the much lower reactivity of the carbohydrates II and III in our experiments opens the question whether more potent antigens may be derived in the future from these types, in this respect like the acetyl polysaccharide from Type I of Avery and Goebel.

Since the de-acetylated derivative was recognized by Avery and Goebel as an artefact, many earlier contradictory statements regarding its chemical and immunological qualities have found an explanation; nevertheless, the complete absence of reactivity in the present experiments of the de-acetylated product is surprising. The question arose whether the effect of the blood group enzyme of feces filtrates upon the acetyl polysaccharide, Type I, and on the group A substance, which according to Freudenberg and Eichel carries acetyl groups, consists in de-acetylation. In this case, acetyl polysaccharide Type I, treated with feces filtrate, should behave like the de-acetylated product. According to the result of Experiment 7, this is not the case, and hence we have to conclude that the change brought about by this treatment is not identical with or analogous to the de-acetylation of the acetyl polysaccharide.

SUMMARY

1. A relationship between the soluble specific substances of pneumococci and the blood group substance A of man can be demonstrated by the inhibition of sheep cell hemolysis by a group specific A-anti-serum. However, there are quantitative differences between the various types.

2. A striking difference exists between the acetyl and the de-acety-

lated polysaccharide of *Pneumococcus* Type I: The de-acetylated carbohydrate fails to react with the group specific A-antiserum, while the acetyl carbohydrate shows a strong reactivity.

3. The minimum amount of the acetyl polysaccharide, which inhibits sheep cell hemolysis by A-antiserum, is almost as small as that of the group specific carbohydrate isolated by Freudenberg and Eichel from urines of group A.

4. The reactivity of the acetyl polysaccharide can be demonstrated not only by the hemolysis inhibition test, but also by complement fixation and by inhibition of group specific iso-agglutination.

5. Feces filtrates, which possess the ability to destroy the blood group specific substances A and B of man, also affect the acetyl polysaccharide of *Pneumococcus* Type I. After incubation with an effective feces filtrate, the acetyl polysaccharide almost completely loses its potency toward the group specific A-antiserum and also its ability to inhibit the iso-agglutination of A blood cells.

6. Acetyl polysaccharide of *Pneumococcus* Type I, having lost its reactivity toward the group-specific A-antiserum after treatment with feces filtrate, still reacts with Type I pneumococcus antiserum which was previously absorbed with de-acetylated polysaccharide, Type I. Thus, the essential effect of the feces filtrate on acetyl polysaccharide, Type I, is not the cleavage of acetyl group, but some other chemical alteration.

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NUTRITIONAL EDEMA IN THE DOG

II. HYPOALBUMINEMIA AND THE AUGMENTATION OF TISSUE FLUID

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Edema in the human being is known to exhibit a variety of manifestations. The regions prone to greatest swelling are not the same in all patients, and in the same patient the swelling often shifts from day to day and sometimes even from hour to hour. In some individuals with marked subcutaneous dropsy effusion into the serous cavities fails to occur or at least does not express itself clinically, whereas in others ascites is marked and edema of the subcutaneous tissues slight or absent. Finally diuresis and loss of edema may occur spontaneously and when no provoking cause is apparent. Because these phenomena and others which characterize edema in the human being have been observed in the dog, because in the dog the events preceding the development of symptoms are capable of being controlled, and because investigators elsewhere may wish to utilize the experimentally induced edema to study other problems, it appears worth while to describe from the symptomatic standpoint the development and course of edema in the group of dogs discussed in the first paper of this series (1). It is evident that caution is necessary in interpreting vagaries of symptomatic expression as due to identical causes in the dog and in man; nevertheless, the very similarity of the vagaries lends support to the belief that the motivating forces are similar in the two species.

Characteristics of the Edema

When dogs are maintained on the protein-deficient diet which was described in the first paper of this series (1) it is generally observed after several weeks that the tissues of the abdominal wall, groin, and extremities have become fuller. At this stage normal body symmetry

is not disturbed and pitting edema is not demonstrable. When true edema does appear after maintenance on the diet for from 1 to 3 months, the development is often sudden and always at a rate disproportionately rapid in relation to that of the previous pre-edematous fullness. The first edema is usually inconstant. It is often present in the afternoon after the animal has stood through the day and absent the following morning after a night in the recumbent posture. With each reappearance the symptom tends to become more marked and daily fluctuations in degree become less striking. With a few animals there has been a noteworthy tendency toward spontaneous and periodic loss of edema. With these animals the symptom has appeared and developed progressively for a week or 10 days and then suddenly, and in association with diuresis, vanished. The subsequent course has been marked by further edema and periodic diuresis. The first edema does not involve all of the body nor all four extremities either equally or simultaneously. Among twenty-two animals the first edema involved only one extremity eleven times, it was present in two extremities seven times, in three extremities once, and in all four extremities three times. It appeared first in one or both hind legs fifteen times and in one or both fore legs three times. That the hind legs are more susceptible than the fore legs is undoubtedly due to the presence of relatively loose subcutaneous tissue about the Achilles tendons and it is here that the earliest signs are generally seen. Edema of the fore legs has always appeared first over the dorsa of the feet and in this region both front and hind legs seem to be equally susceptible. As the process progresses all four extremities usually become involved in the dropsy. Among twelve dogs in which the edema began in the hind legs, involvement of the fore legs had appeared within 3 to 14 (average 7) days. In two instances the fore legs remained free of edema even after the process had extended to otherwise generalized anasarca with massive ascites. That the fore legs are usually involved sooner or later has been mentioned specifically because in our experience these extremities have been spared in the acute edema which follows plasmapheresis. With further passage of time the edema tends to become more and more massive, the toes become swollen and spread apart, the swelling extends upwards to involve the extremities through their entire length, and finally bag-like sacks of

edematous tissue, sometimes resembling mammary glands, may be seen hanging from the chest and abdominal wall. In several instances edema of the scrotum has been massive, now and again the face and eyelids have appeared swollen, and in one case there was marked chemosis of the bulbar conjunctivae. Ascites, which may or may not be present, will be discussed presently.

The experimentalist who plans to study this form of edema may wish to know whether all animals which are maintained on the diet develop the symptom, the time required for its appearance, and the time during

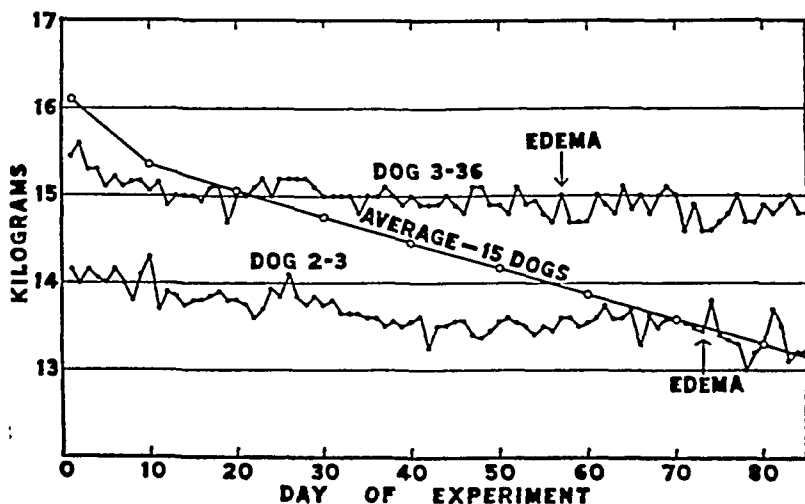


CHART 1. Behavior of body weight during maintenance on low protein diet.

which observation and study are possible. To date twenty-eight dogs have been started on the diet and of these twenty-two developed edema. Of the remaining six experiments five were terminated prematurely, three times because the animals contracted distemper and twice because of anorexia with refusal to eat the diet. In a single instance only edema had not developed after a hundred days of maintenance on the diet and the animal, which had developed severe jaundice, was sacrificed. In this case, however, the serum albumin, although it had declined progressively, had not fallen to a level which was reached with many other dogs before edema appeared. Among twenty dogs whose course was not complicated the edema appeared

in from 35 to 100 days or in an average of 60 ± 15 (S. D.) days. One might expect that the interval could be shortened by initial periods of plasmapheresis. In two instances in which this was tried, the attempt was not successful and edema developed after 53 and 60 days, respectively. The period of survival after the appearance of edema or more properly the time over which edema has persisted and been available for study cannot be evaluated accurately from our data because the course has been complicated by various experimental procedures which have included transfusions, diuretics, and changes in diet. Some of the animals have been sacrificed and some have been allowed to recover. With these limitations in mind it may be stated that edema was present in the twenty-two dogs from 1 to 77 days or for an average time of 27 ± 17 (S. D.) days. In individual animals it is possible with experience to estimate the period of survival more closely from consideration of the physical state, the appetite, and the serum protein concentrations. Experimental studies can be planned accordingly.

Behavior of the Weight Curve

In the majority of experiments the animals have exhibited a progressive decline in weight during the period of maintenance on the diet, the loss being greatest during the first week or 10 days and thereafter more gradual. The extent of the loss can be gauged from Chart 1 in which has been plotted the average weight curve of fifteen dogs which received the diet containing cod liver oil. Inasmuch as the food consumed by most of the dogs was deficient both in nitrogen and total calories, we have thought the loss of weight to be due to destruction both of body protein, *i.e.* muscle, and of body fat. The destruction of tissue protein is proved by the consistently negative nitrogen balance (1). That loss of body fat has also contributed to the decline in weight is suggested by the repeated observation with individual animals of a rapid loss in weight following voluntary reduction in the quantity of diet consumed and by the fact already reported that such reduction does not greatly affect the magnitude of the nitrogen loss. During periods when the appetite has remained normal and the total diet has been consumed, we have been impressed again and again by the absence of conspicuous loss of weight. In an experiment which

was described in an earlier paper (2) observations extending through 42 days before edema had developed revealed a loss in weight of 620 gm. During the same period studies of the nitrogen balance indicated the destruction of 1059 gm. of muscle. In two additional experiments, the details of which are given in Table I, studies of nitrogen balance again showed that the animals lost less weight than would have been expected from the magnitude of the nitrogen loss. With two more animals, whose weight curves are shown in Chart 1, the appetite re-

TABLE I
Comparison between Observed Loss of Weight and Loss of Weight Calculated from Negative Nitrogen Balance

Dog 8-4a Initial weight 15.68 kg.			Dog 8-06 Initial weight 15.45 kg.		
Metabolism period	Calculated weight loss	Observed weight loss	Metabolism period	Calculated weight loss	Observed weight loss
days	gm.	gm.	days	gm.	gm.
1-6	338	380	1-7	328	320
7-13	350	480	8-14	283	240
14-19	270	20	15-21	265	180
20-25	153	190	22-28	259	-120
26-32	217	140	29-35	265	20
33-39	200	-250	36-42	171	80
40-46	226	150	43-49	253	290
47-53	185	90	50-56	250	410
Total.....	1939	1200	Total.....	2074	1420
Edema appeared on 65th day			Edema appeared on 92nd day		

mained good and the loss in weight was so slight as to suggest at once a discrepancy with nitrogen loss even though the latter was not measured. It may be mentioned that the serum proteins of these animals declined as rapidly as in other experiments and that no correlation has been found between the rapidity of the fall in serum albumin and either the weight loss or the quantity of food consumed. Under the circumstances, we have interpreted the discrepancy between the weight loss calculated from nitrogen balance and that actually observed as due to a continual retention of water during the period of

pre-edema. It is realized that such a discrepancy would also occur if fat were being stored while tissue protein was undergoing destruction and it is true that many of our animals have shown at autopsy abnormal infiltrations of fat in the liver. Nevertheless, the panniculus and omental fat have never been more abundant than in normal animals and indeed these stores have usually appeared to be depleted to such a degree as to suggest a considerable loss rather than an increase in total body fat. Furthermore, physical signs of pre-edema which were described in the last section favor the interpretation that has been given. It is interesting as well as confirmatory that a similar observation was made by Bischoff and Voit (3) as long ago as 1860. These investigators maintained a dog exclusively on bread for a period of 41 days. They found that although the nitrogen loss corresponded to 3,717 gm. of muscle, the animal lost only 531 gm. in weight, and concluded that water retention must have taken place. In other experiments in which cats were fed exclusively with bread they found the water content of the muscles and brain to be from 3 to 4 per cent higher than in control cats fed with mixed food.

In the majority of experiments the development of edema was not preceded by definite fluctuation in the weight curve. In a previous publication (2) this fact was noted and interpreted to mean that the state of pre-edema gradually merged with that of true edema in such a way that sudden accumulation of fluid in the tissues did not occur. Subsequent observations have shown that this interpretation was at least partly in error. For reasons given in the preceding paragraph we are still convinced that fluid does accumulate in the tissues for some time preceding the appearance of palpable edema; nevertheless, from inspection alone it has become clear that the development of palpable edema represents a sudden local increase in the rate of accumulation.¹ It seems likely that the amount of fluid required to

¹ Because they believed that the onset of edema was not associated with a sudden increase in the rate of accumulation of fluid in the tissues, Weech, Snelling, and Goettsch (2) suggested that the term "correlation level" rather than "critical level" be used to refer to serum protein concentrations below which edema is usually present and above which it is not. From the above discussion it appears that the reason for the suggestion was not valid and the expression "correlation level" will not be used in this report.

produce dependent edema in one or two extremities of the dog is too small to produce an obvious change in body weight. It may be that the phenomenon is associated with an internal shift in the distribution of fluid already in the body inasmuch as studies of salt metabolism have failed to indicate a significant retention of either sodium or chloride at this time. In either case the fact that palpable edema does not develop in all regions of the body at the same time but rather by successive steps over a period of several weeks, tends to obscure whatever effect there might be on the weight curve.

In one circumstance, however, the development of edema may be associated with a marked rise in body weight; namely, when fluid is accumulating in the peritoneal cavity. In one instance the rise began before subcutaneous edema had appeared; in six other instances it followed within 1 to 15 days after palpable edema had first been demonstrated. When ascites is fully developed the total gain in weight is sometimes enormous. Dog 1-91 showed an increase from 9.85 to 12.95 kilos over a period of 13 days, the increment of 3.10 kilos representing 31.5 per cent of the previous body weight.

Edema and the Serum Proteins

The concentrations of albumin, globulin, and total protein in the serum at the time of the first appearance of palpable edema are shown in Table II for twenty-one animals. With albumin all values are below the range of normal variation and are distributed between 1.04 and 2.17 gm. per cent. The globulin values are normal so that the reduction in total protein obviously results from the albumin deficits alone. However, although the total protein is usually reduced below normal levels, concentrations within the range of normal are not infrequent, and a satisfactory correlation cannot be drawn between it and edema. A positive correlation with edema exists for the albumin fraction only but even in the case of albumin the variations encountered among different animals are fairly wide. To investigate the possibility of defining the critical level for edema in less variable terms, we have calculated both by the formula of Wells and his associates (4) and by the method of Govaerts (5) the colloid osmotic pressure of the several sera. The results are included in Table II. The coefficients of variation for either of the osmotic pressure tabulations

and for albumin alone do not differ significantly. Evidently the critical levels vary just as widely when expressed in calculated units of osmotic pressure as in grams per cent of albumin. Unfortunately,

TABLE II

Serum Protein Concentrations Associated with First Appearance of Edema

Dog No.	Albumin per 100 cc.	Globulin per 100 cc.	Total protein per 100 cc.	Colloid osmotic pressure	
				Wells	Govaerts
	gm.	gm.	gm.	mm. H ₂ O	mm. H ₂ O
8-4a	1.04	1.93	2.97	82	116
2-06	1.04	2.94	3.98	110	136
1-91	1.15	2.48	3.63	102	135
8-4b	1.29	2.48	3.77	109	146
8-4c	1.29	3.32	4.61	134	162
8-38	1.30	2.61	3.91	114	149
8-06	1.35	2.99	4.34	127	160
3-87	1.37	2.13	3.50	103	145
2-07	1.38	2.55	3.93	116	154
5	1.40	2.00	3.40	101	145
1-90	1.47	2.90	4.37	131	167
2-3	1.52	1.90	3.42	104	152
4-49	1.59	4.06	5.65	174	199
5-89	1.63	2.80	4.43	137	178
3-36	1.75	3.12	4.87	155	193
4-30	1.79	1.97	3.76	120	173
2-93	1.79	3.22	5.01	160	198
5-97	1.85	3.16	5.01	162	201
1-89	1.95	2.51	4.46	147	196
2-05	1.95	3.53	5.48	180	216
5-65	2.17	2.70	4.87	167	216
Average.....	1.53	2.73	4.26	130	168
S.D.*.....	0.31	0.56	0.70	27.1	27.9
C.V.†.....	19.9	20.4	16.5	20.8	16.6

* S.D. = standard deviation of average.

† C.V. = coefficient of variation.

the calculation depends upon formulae which were derived from measurements of human serum. Analogous formulae for canine serum do not exist. It is possible, therefore, that a narrower range of varia-

tion in the critical levels may be demonstrated when direct measurements of the osmotic pressure have been made. At the present time, however, it is reasonable to assume that the levels actually are quite variable and to suspect the existence of other factors of importance in determining the exact time of appearance of the first edema. That other factors must exist is apparent when it is recalled that a considerable interval may intervene between the onset of edema in the fore legs and in the hind legs in the same experiment, a circumstance which means that different critical levels might be recorded for the first appearance of edema in different regions of the same animal.

From the standpoint of the investigator who wishes to utilize the method of diet for producing experimental edema, it is apparent that in general the time (T) which must elapse before edema appears will be short when the edema develops in association with a high concentration of serum albumin and relatively long when the critical level (A_c) for the individual animal is low. The extremes of our experience show that Dog 5-65 developed edema after 40 days of maintenance on the diet and in association with a serum albumin concentration of 2.17 gm. per cent, and that in contrast 100 days had elapsed and the serum albumin had fallen to 1.04 gm. per cent before Dog 2-06 became edematous. One would also expect to find some degree of correlation between the albumin concentration at the start of the experiment (A_s) and T ; that is, it should require a greater time to deplete the albumin to edema levels when the initial concentration is high. Actually a positive correlation has been demonstrated between T and A_s , but an analysis of our data by the method of partial correlation indicates that A_s was approximately twice as important as A_c in determining T . On the average, lowering the initial level of albumin by 1 per cent decreased by 11 days the time required for producing edema, whereas lowering the critical level by 1 per cent increased the time by 23 days. However, the coefficient of multiple correlation, which measures the combined effect of A_s and A_c on T was only 0.49 and it is evident that other unknown factors were operative in determining T . Among them we have considered the possible influence of the original nutritional state, the amount of weight lost during the experiment, and the quantity of food ingested, but have failed to demonstrate that importance can be attached to these factors.

When the serum albumin is raised by appropriate measures the edema will disappear. When the rise follows interruption of the low protein diet and return to adequate feeding, the elimination of edema usually occurs in association with concentrations of serum albumin within the same range as that described for the first development of edema. Now and again, however, conditions have been encountered under which the edema has not disappeared until the albumin had regenerated considerably above this range. An experiment which exemplifies this happening was illustrated in Chart 5 of the first paper of this series (1). In the experiment Dog 3-36, which developed edema in association with an albumin concentration of 1.75 gm. per cent, did not become free of edema until an adequate diet had been offered for 19 days and until the level of albumin had risen to 2.75 gm. per cent. In two instances in which discrepancies of this magnitude existed between the critical levels for the appearance and disappearance of edema, the dropsy had existed for more than a month when the change in diet was instituted. In other experiments in which the albumin concentration has been raised abruptly by means of transfusion we have not always observed an immediate disappearance of edema with concentrations above the critical level.

Protein Content of Edema Fluids

Thirty samples of edema fluid from the subcutaneous tissue of the extremities of fourteen dogs have been analyzed for total protein. The results appear in Table III. The range of protein concentration was from 0.02 to 0.72 gm. per cent. The average protein level was 0.230 gm. per cent and the median level was 0.165 gm. per cent. The discrepancy between the average and median values indicates the absence of normal frequency distribution in the results. Actually in five instances only, or one-sixth of the total number, was the concentration greater than 0.35 gm. per cent. Table III also shows the contemporaneous protein content of the serum and the number of days which had elapsed after the onset of edema before the fluids were collected. With neither of these factors has it been possible to demonstrate a significant positive correlation with the amount of protein in the edema fluid, a fact which is roughly apparent from the manner of arrangement of the data in the table. Apparently, some other factor,

presumably the permeability of the capillary, is of such importance in determining the protein in edema fluid as to obscure the effect of variations in serum protein and it would seem that the circulation of edema fluid through the lymphatics is sufficient to offset the gradual increase in protein content which would be expected if the fluid remained dormant in the tissues.

TABLE III
Comparative Data Which Show Lack of Relationship between the Protein of Edema Fluid and the Protein of Serum or the Duration of Edema. Data Arranged in Ascending Order of the Protein Content of Edema Fluid

Dog No.	Duration of edema	Protein of serum per 100 cc.	Protein of edema fluid per 100 cc.	Dog No.	Duration of edema	Protein of serum per 100 cc.	Protein of edema fluid per 100 cc.
	days	gm.	gm.		days	gm.	gm.
Subcutaneous edema fluid				Subcutaneous edema fluid			
8-4b	14	2.88	0.02	3-36	29	4.64	0.23
8-4b	14	2.88	0.04	5	2	3.24	0.23
8-06	7	3.53	0.06	5-89	17	3.76	0.28
8-06	7	3.53	0.07	5-97	9	4.68	0.28
8-38	16	3.29	0.08	5-65	14	4.73	0.29
2-07	41	3.63	0.08	3-36	15	5.17	0.33
5-97	23	5.03	0.10	4-51	26	4.61	0.34
1-90	40	4.32	0.10	5	1	3.24	0.36
1-90	40	4.32	0.11	2-3	16	3.38	0.48
1-90	40	4.32	0.12	2-3	16	3.38	0.56
8-06	5	3.77	0.13	2-93	12	4.43	0.71
8-06	11		0.15	2-93	23	4.72	0.72
2-06	6	4.03	0.16	Ascitic fluid			
5-97	23	5.03	0.16	2-07	44	2.57	0.01
2-3	15	3.38	0.17	8-4b	13	2.88	0.02
2-3	15	3.38	0.21	2-07	41	3.63	0.04
5-89	18	3.69	0.22	5-97	30	4.50	0.13
3-36	22	4.69		5-89	25	3.60	0.72

The protein content of five samples of ascitic fluid is included in Table III. In four instances the level was less than 0.15 gm. per cent and in one instance greater than 0.70 gm. per cent.

Ascites

The accumulation of fluid in the peritoneal cavity in amounts sufficient to produce a noticeable increase in body weight and signs of

ascites on physical examination was observed seven times in the group of twenty-two dogs which developed subcutaneous edema. Because the majority of animals did not exhibit the symptom the protocols were examined with a view to discovering provoking circumstances. The examination showed that in five instances out of the seven the first signs of ascites followed closely an abrupt increase in the amount of salt and water in the diet. On numerous other occasions, however, similar increases failed to evoke the same response. In two instances only, the ascites developed when the salt intake had been constant at a level of approximately 5 gm. daily for longer than 2 weeks. The results are at least understandable if it is supposed that a sudden increase in dietary salt produces a stress throughout the tissues which favors distention of the intercellular spaces and accumulation of fluid. If the stress is exerted when the tissues possess a reasonable reserve strength retention will not result. When the reserve is broken fluid will accumulate in the organism. In a general way it has seemed that subcutaneous tissues and peritoneal cavity react differently to the application of stress. When the stress is increased gradually and exerted constantly or repeatedly over long periods the subcutaneous tissues yield more readily than the peritoneum. When the stress is applied suddenly the reverse is true,—the subcutaneous tissues are generally resistant whereas the peritoneal cavity, once the barrier to the accumulation of fluid has been broken, does not manifest further resistance for some time and large collections can and do develop rapidly. Although a sudden stress of this nature seems to result when the salt in the diet is increased abruptly, there are undoubtedly other factors or processes capable of effecting similar shifts in the stress on the tissues.

The difference in behavior between subcutaneous tissue and peritoneal cavity is exemplified further by contrasting the form of edema which develops rapidly in plasmapheresis experiments with that produced slowly by restriction of protein in the diet. In plasmapheresis experiments ascites is invariably present whereas edema of the extremities is less intense than in nutritional experiments. If the plasmapheresis experiment is so arranged that fluid retention, although great, is limited to a period of 24 hours or less, the contrasting responses of subcutaneous tissue and peritoneal cavity are even more striking.

Such an acute retention can be obtained by maintaining the dog on a salt-free diet during the 8 or 10 days required for depleting the serum albumin below the critical level, and then administering by gavage in the course of a few hours large volumes of physiologic saline solution. The details of an experiment are presented in Table IV. Although in this instance the final abrupt rise in weight indicated that more than $2\frac{1}{2}$ liters of fluid had been retained after a period of about 18 hours,

TABLE IV
Plasmapheresis Experiment

Dog 9-92.

Day of experiment	Blood exchanged by plasmapheresis	Salt addition to diet	Body weight (9 a.m.)	Remarks
	cc.	gm.	kg.	
1	350	0	14.80	Serum albumin = 3.06 gm. per cent
2	380	0	15.30	
3	420	0	15.15	
4	380	0	15.30	
5	490	0	15.55	
6	300	0	15.45	
7	520	0	15.25	
8	575, 300	22.4	15.35	
9	—	—	18.00	Serum albumin after second exchange = 0.85 gm. per cent Weight gain 2.65 kg. in 18 hrs. Subcutaneous tissue everywhere fuller. Mild edema of abdominal wall. No edema of extremities. Massive ascites; at autopsy in late afternoon 1100 cc. fluid withdrawn from peritoneal cavity. Total protein of ascitic fluid = 0.01 gm. per cent

pitting edema of the extremities did not develop and the peritoneal cavity was the only large depot which could be demonstrated easily. Of significance, however, is the fact that the subcutaneous tissue everywhere was fuller, obviously contained more fluid, but had not yielded to the point of exhibiting palpable edema. It is precisely this peculiarity of resistance to acute stress on the part of tissues which yields readily to prolonged stress which contrasts sharply with the behavior of the peritoneal cavity.

DISCUSSION

A description of the events which attend the development of nutritional edema in the dog has been the main purpose in presenting the data contained in this paper. It is desired here merely to modify and elaborate briefly concepts, previously expressed (2), of the mechanism involved in the formation of edema. Those forces which operate on the inside of the wall of the capillary,—colloid osmotic pressure and capillary blood pressure,—have been discussed frequently in recent literature. Under conditions of rest and at times when the volume of fluid in the interstitial spaces is constant these forces must be in equilibrium with others on the outside of the capillary. The extramural forces may likewise be divided into osmotic and mechanical components. Of these, in the type of edema under consideration, the effective osmotic pressure is relatively small and discussion at this time will, therefore, be confined to the other factor, mechanical pressure in the interstitial spaces. The effect of variations in this pressure on the volume of fluid in the spaces must obviously depend on the physical properties of the connective tissue framework which encloses the spaces. If the intercellular tissue were composed of a rigid non-yielding substance it is clear that no amount of pressure could result in distention and the accumulation of edema. If the tissue were perfectly elastic, equal increments of stress would result in equal degrees of stretching and corresponding increments in the volume of interstitial fluid. Obviously the tissue spaces are neither non-yielding nor perfectly elastic. The observations described in this paper indicate that the increasing stress on the tissues, which results from the declining level of albumin in the blood, does lead to expansion of the intercellular spaces for some time prior to the appearance of edema. Consideration of the quantitative relationship between weight loss and nitrogen loss as well as physical signs of pre-edema both suggest retention during this period of moderate amounts of fluid. The development of palpable edema, however, takes place suddenly in a manner which suggests that increasing stress has finally become too great to be tolerated by the elastic strength of the tissue framework. The tissues seem to be torn apart and edema fluid accumulates rapidly throughout the area involved. For the time being the barrier

to accumulation is removed and the volume of transudate is determined by the extent of the involved area, by the residual strength in surrounding tissues, and finally by back-pressure from overlying skin.

In a previous paper on the relationship between lymph flow and edema (6) it was pointed out that massage, passive movement, or voluntary motion activate the pumping mechanism of the lymphatic valves, that the activation results in removal of fluid from the tissues through the lymphatics, and that mechanical pressure in the extracapillary spaces must be lowered by the process. It is, therefore, apparent that, under normal circumstances of alternate rest and activity, pressure within the tissue spaces varies in a periodic way. The higher values which under appropriate conditions lead to separation of the spaces and the accumulation of edema occur only during rest or when physical activity is extremely reduced. The magnitude of the pressure required to accomplish this effect is not known although it is clear that the force cannot be greater than capillary blood pressure. Higher pressures by collapsing the capillaries would automatically stop the process of filtration. It is further clear that those measurements which have been attempted of pressure within the tissue spaces and in which the relation between the time of measurement and previous physical activity was not controlled (7) can have no meaning in evaluating the forces involved in edema formation.

If the concept outlined in the preceding paragraphs is correct it follows that the critical level of protein in the serum is determined by that concentration which permits the attainment in the tissue spaces of mechanical pressures great enough to break down the restraining action of the connective tissue boundaries of the spaces. Obviously such pressures can arise only when the colloid osmotic pressure of the serum has fallen below capillary blood pressure, but the effect of the fall on pressure outside the capillary is more directly the cause of edema than the disturbance of the balance between the intramural forces. Because physical activity as well as the relationship between the intracapillary forces both have an effect on pressure in the tissue spaces it is apparent that the degree of spontaneous activity exhibited by an animal will influence the exact level of the serum protein which will be regarded as critical. We have already reported the fact (6) that a previously developed edema can be eliminated by an enforced

régime of activity. The reason for the elimination, however, is complicated by the fact that veins as well as lymphatics are furnished with valves and that the function of both is stimulated by muscular motion. When the veins are filled by hydrostasis with a column of blood, stimulation of their valves leads to a reduction in capillary blood pressure, and this effect will be added to that of the lymphatics in removing fluid and lowering pressure in the tissue spaces.

The concept which places primary importance on the elastic strength of the connective tissue framework of the tissue spaces in determining the site and time of appearance of edema provides a basis for understanding several of the phenomena which have been described. The singular susceptibility of the loose tissue about the Achilles tendons contrasts sharply with the resistance exhibited by the firmer tissue over the dorsa of the feet. It is unlikely that the difference in reaction can be explained by variations in capillary blood pressure for the two regions are anatomically contiguous and exposed to the same hydrostatic force. The concept allows a clear comprehension of the distinction between pre-edema and palpable edema in accordance with the discussion in a preceding paragraph. It assists in an understanding of the difference in protein levels which may be critical for the formation and disappearance of edema in the same animal. For it is conceivable that after marked edema has disturbed the integrity of the tissue spaces time must elapse and a new level of pressure conditions arise before the edema is removed. Finally there is provided a comprehensible basis for the phenomenon described in the section on ascites; namely, that a sudden fall in the colloid osmotic pressure of the serum does not have the same effectiveness on subcutaneous edema formation as a gradual decline over a number of weeks which is associated with progressive malnutrition.

SUMMARY

The manner in which edema develops in dogs maintained on a diet low in protein is described. Pre-edematous fullness of the tissues is observed for some weeks before palpable edema develops. The state of pre-edema does not merge gradually with that of true edema but rather the transition is relatively sudden. Among twenty dogs the

time required for the production of edema varied from 35 to 100 days and averaged 61 days.

With three animals in which the nitrogen metabolism was studied during the period before edema had developed, the observed loss of weight was consistently less than the theoretical loss of weight calculated from the negative nitrogen balance. Reasons are given for interpreting the discrepancy as evidence of increasing retention of fluid during the stage of pre-edema. In general the weight curve does not rise during the transition from pre-edema to edema. However, the weight does increase rapidly when fluid is accumulating in the peritoneal cavity.

The level of serum albumin which is critical for the development of edema varied between 1.04 and 2.17 gm. per cent. The range is sufficiently wide to suggest the existence of other factors of importance in determining the exact time of appearance of edema. During the phase of recovery the level of albumin which is critical for the disappearance of edema may be appreciably higher than the level which was critical for the formation of edema.

Among thirty samples of edema fluid the protein concentration was from 0.02 to 0.72 gm. per cent. The average protein level was 0.230 and the median level 0.165 gm. per cent. A positive correlation is not demonstrable between the duration of edema and the protein content of edema fluid.

A difference in behavior toward fluid retention between subcutaneous tissue and peritoneal cavity is pointed out. Subcutaneous tissue is more resistant to acute stress and less resistant to prolonged or repeated stress than the peritoneal cavity.

The rôle of tissue pressure in the etiology of edema is discussed. It is suggested that the critical level of protein in the serum is the concentration which permits the attainment in the tissue spaces of mechanical pressure great enough to break down the restraining action of the connective tissue boundaries of the spaces.

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STUDIES ON BACTERIAL LOCALIZATION
EFFECTS OF SPECIFIC IMMUNIZATION AND OF A GUM ACACIA MEDIUM
ON LOCALIZATION OF TYPE I PNEUMOCOCCI IN MICE

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PLATES 35 to 37

CORRECTION

In Vol. 61, No. 5, May 1, 1935, page 643, fourth line from the bottom, for

read

($C_{14}H_{18}O_3$ or $C_{14}H_{17}O_2OH$)

($C_{14}H_{18}O_3$ or $C_{14}H_{20}O_3$)

generalization when initiated by the actual infecting agent, experiments have been conducted to determine the effects of local inflammation or cellular mobilization following the application or introduction of irritants at various intervals before infection. These experiments have shown that infections can be localized in prepared fields, such as the pleural and peritoneal cavities, when they cannot in the tissues of normal animals (1). Further, localization occurs in the presence of a barrier of macrophages when it does not if the barrier is composed chiefly of polymorphonuclear leukocytes (2). Even macrophages may not prevent bacterial dissemination, however, unless the organisms have been previously sensitized. Clark (3) has shown this for pneumococcal infection of the pleural cavities of rabbits. Thus non-specific cellular mobilization in a normally susceptible animal cannot alone localize the pneumococcus; specific antibody is an essential accessory factor.

Although it is generally agreed that an effective weapon against certain pathogens can be acquired through specific active or passive immunization, opinions differ as to the exact means by which bacteria are thereby localized. Some hold that fixation is secured and maintained by humoral factors involving local extracellular reaction between antibody and living antigen, in a manner analogous to that observed by Opie (4) in the localization of soluble antigens. If, however, cells are mobilized, one must consider the modification of their phagocytic activities resulting from the presence of antibody. Mudd and his associates (5) have shown that cohesiveness, agglutination, opsonization and phagocytosis of bacteria are correlated in specific immunity. From histologic studies of infected tissues after specific active immunization, Cannon and Pacheco (6) have emphasized that fixation and agglomeration of bacteria at the site of introduction are important aids to phagocytosis.

What part in specific immunity is played by the accelerated, more extensive and more locally destructive inflammatory reaction of hyperergy or hypersensitiveness? This reaction may, as in massive reinfection of a tuberculous host, actually promote dissemination of the bacteria. Significant work by Rich and his associates showed that under experimental conditions immunity is not dependent upon "allergy" (the hyperergic reaction) and can be dissociated from it. Simultaneous introduction of pathogenic bacteria and irritants heightening the inflammatory response favored dissemination of the organisms (7, 8). Animals actively or passively immunized against pneumococci or other organisms were protected on introduction of homologous living bacteria in the presence of less inflammation than occurred in normal controls; here reaction between antibody and intact bacterial antigen did not result in allergic inflammation (9-11). When rabbits were protected by a reaction involving accelerated and more widespread appearance of exudation, edema and necrosis, the allergic reaction was regarded as of minor importance. Greatest emphasis was placed on adherence of bacteria to each other and to the tissues at the site of introduction within 30 minutes after infection (10). Rich believed that in the case of the pneumococcus, there is involved primarily a specific agglutinative process which acts independently of allergy and is in operation before any inflammation appears (8).

Can bacteria be agglomerated *in vivo* by other than specific means? If so, will this maintain fixation and promote phagocytosis? Reimann (12) was led to a study of the effect of increased viscosity of the medium on bacterial clumping from a consideration of certain features of infectious diseases. He pointed out that fever is often accompanied by increased fibrinogen and total globulin concentration in the blood plasma, with an accompanying elevation of viscosity. He showed that immunagglutination of pneumococci and Friedländer's bacilli occurred at distinctly higher dilutions in the more viscous plasma of patients with fever, as compared to that in normal plasma. Solutions of gum acacia (gum arabic) also accentuated specific agglutination. Best results were obtained at viscosities 3.5 to 5.6 times that of distilled water. Concentrations greater than these apparently inhibited clumping. Various other viscous substances, such as solutions of gelatin, egg white, starch and agar, were less effective. Finally, Reimann studied smears made from peritoneal fluid up to 1 hour after intraperitoneal injection into mice of Type I pneumococci. With acacia present in the inoculum, there was clumping of host cells but not of bacteria. If a small amount of immune serum had been added, however, the cells and bacteria were tightly clumped together. An equal amount of immune serum caused no clumping in a saline medium. In other words, although agglutination of bacteria was not enhanced by solution of acacia alone, it was definitely increased by acacia together with immune serum.

In the present re-examination of the cellular and specific humoral reactions to bacterial infection, we have made morphologic studies of the abdominal walls of mice at frequent intervals after infection with Type I pneumococci. To facilitate observations of gross lesions, the subcutis was chosen as the site of inoculation. We have been interested especially in the answers to these questions: Is the hyperergic reaction an essential part of acquired immunity? In bacterial localization and destruction, what is the relative importance of agglutination and of phagocytosis? What influence on the course of infection is exerted by a viscous gum acacia medium, used either with or without specific antibody?

Methods

Young adult male white mice weighing about 20 gm. were used. The Type I pneumococci employed¹ had been used in this laboratory in connection with experiments on rabbits for about a year. Their virulence for mice was exalted and maintained by repeated passage through mice. 24 to 48 hours before injections, the abdominal walls of the mice were epilated, using a barium sulfide paste. The cocci from a 12 to 24 hour blood agar slant were suspended in 5.0 (in a few cases 2.5) cc. of sterile 0.85 per cent solution of sodium chloride; of this, 0.1 cc. of a 1:10 dilution was injected into the subcutis of the anterior abdominal wall. No exact determination of virulence was made. When introduced in this amount, the pneumococci caused the death of normal mice with rare exceptions in 48 hours, often within 24.

A formalized vaccine was prepared from 18 to 24 hour cultures of Type I pneumococci on blood agar in Kolle flasks, by suspension of the organisms in saline containing 0.4 per cent formalin. The sterility of the vaccine was checked before and during use. Immune serum was obtained from rabbits after repeated intravenous administrations of the vaccine. The serum was protective for mice and by the usual macroscopic method agglutinated the homologous organisms *in vitro* to a dilution of 1:320.

Solutions of gum acacia were prepared from the crude commercial product and filtered through cotton. The bacteria were carried to the final dilution in salt solution and then placed in acacia of such concentration as to make a 10 per cent solution of acacia in saline after addition of the organisms. In *in vitro* experiments, immunagglutination progressively increased with greater concentrations of acacia up to 5 or 6 per cent; with higher concentrations, it fell off sharply. For injections, 10 per cent acacia was used to allow for dilution *in vivo*. At room temperature (28°C.) and without control of the pH, the relative viscosities of 5 and 10 per cent solutions of acacia were 4.24 and 8.50 respectively (Hess capillary viscosimeter).

A control group of normal mice received pneumococci in saline as described.

For a study of the relationship between immunity and hyperergy, three groups of mice were used. Those of the first group were actively immunized by 5 or 6 intraperitoneal injections of 0.5 cc. of vaccine at 4 day intervals. The living organisms were injected 10 to 16 days after the last vaccination. Members of another group were passively immunized by introduction of 0.5 cc. of immune serum intraperitoneally daily for 3 days. The cocci were injected subcutaneously on the following day. Mice of the third group were first vaccinated intraperitoneally as above. In addition, they received 0.02 cc. of the vaccine subcutaneously in adjacent parts of the anterior abdominal wall, either 4 times at 4 day intervals or daily for 5 days, that is, until reacting to vaccination with rapid abscess formation. Live pneumococci were injected 7 to 15 days later.

¹ The Type I pneumococci were obtained from Dr. O. H. Robertson.

Experiments with solution of acacia as the medium were of three types. Normal mice of one group received pneumococci suspended in 10 per cent acacia. To determine the effects of a small amount of immune serum, we inoculated mice of a second group with pneumococci suspended in 10 per cent acacia together with 0.001 cc. of immune serum; a control group was treated similarly except that saline was substituted for acacia. The quantity of immune serum used, in attempting to ascertain maximum differences dependent upon the use of an acacia medium, was determined by survival experiments. 0.01 cc. of immune serum injected together with the cocci protected irrespective of the medium used. 0.001 cc. of immune serum protected over three-fourths of the mice receiving pneumococci in acacia, and less than one-fourth of those inoculated with the saline suspension. Smaller amounts of immune serum failed to protect with either medium. Finally, mice of a third group were treated so as to exhibit the effects of immune bodies in the tissues rather than in the acacia inoculum. 1 month after local active immunization, during which 0.1 cc. of vaccine was injected subcutaneously into adjacent parts of the abdominal wall daily for 5 days, living pneumococci were introduced in the acacia medium. A control group similarly prepared received a saline suspension.

Some of the mice in each of the described groups were set aside for survival experiments. Inoculated mice were at first observed for 30 days after infection, later for 10 days, since time of death when it occurred and duration of gross lesions were well within the latter period. From each group, mice were killed and the tissues of the anterior abdominal wall were taken for histologic study 5, 15 and 30 minutes and 1, 2, 3, 4, 6, 9, 12, 15, 18, 24, 36 and 48 hours after infection, and when survival made possible, at 72 and 120 or 144 hours. The tissues were attached to cork rings and immersed at once in warm Zenker-formol. The celloidin used for embedding was allowed to harden very slowly in covered dishes to facilitate cutting, as suggested by Bloom (13). From portions of the abdominal wall 1.0 to 1.5 cm. in diameter, serial sections 7 or 10 microns thick were cut parallel to the cutaneous and peritoneal surfaces. Sections were stained with Maximow's hematoxylin-eosin and Azur II or the modified Gram stain of Brown and Brenn (14). From most groups, mice were killed 5 and 15 minutes after injection, and with fluid aspirated from the blebs at the inoculation sites, smears were made and stained with the Giemsa stain in Haden's buffered solution.

RESULTS

1. Control Group.—The bleb caused by inoculation disappeared within $\frac{1}{2}$ hour. For 12 hours after the injection into normal mice of a saline suspension of pneumococci, there were no changes visible in the gross at the site of injection. At later stages, there were hyperemia and moderate thickening of the abdominal wall. Edema was not nearly as conspicuous in the gross as that described (Rhoads and Goodner (15) and others) in the dermal pneumococcal infection of rabbits. Most of the mice killed at 12 hours and later appeared sick (signs such as ruffled hair,

crusted eyes, labored respiration, trembling, staggering). None survived for section at 72 hours.

Microscopically, there was no significant localization of the organisms. Pneumococci were from the first scattered extracellularly in the loose connective tissue (Fig. 1). Phagocytosis was negligible, and proliferation of the cocci was apparently little hindered. A slight proliferation of the local cells of the subcutis occurred in the early hours. An exudate composed chiefly of polymorphonuclear leukocytes appeared at 1 hour, and 2 hours after infection had increased sufficiently to obscure the normal structures of the subcutis. 12 hours after infection, there was considerable edema fluid in the subcutis. At the 15 hour stage cocci were present in moderate numbers at the parietal peritoneum. In later stages, the exudate and edema fluid, with innumerable bacteria carried along in the latter, spread laterally to include most of the region examined, and involved the deeper portion of the dermis, the muscle layers, subperitoneal tissues and parietal peritoneum. At 48 hours there was a well developed fibrinopurulent peritonitis. The subcutaneous lymphatics were patent, dilated and often contained many lymphoid cells during the early hours. At 36 hours some were occluded by fibrin plugs, in the thickened subcutis where greatest exudation and necrosis occurred. Fibrin in the tissue spaces was not seen before the 12th hour, and not in large amounts until the 36th.

2. Immunity and Hyperergy.—

(a) *Group Intraperitoneally Vaccinated.*—Histologic changes in the abdominal wall following intraperitoneal vaccination were definite. At the time when these tissues were first examined microscopically, 10 to 16 days after the final vaccination, there were many medium sized and large mononuclear phagocytic cells in the subcutis. These cells were scattered and in small nests (Fig. 11), without mitotic figures. The size of the larger was about that of a fibroblast nucleus. The nucleus of the mobilized cell type, often excentric, was rich in basichromatin, staining darker than a fibroblast nucleus. Nucleoli if present were obscured. The cytoplasm stained pale blue to blue-gray with the Maximow stain, and contained scattered granules and vacuoles. Whether mobilization of these cells represented part of a generalized mesenchymal reaction is uncertain, for tissues from other parts of the body were not examined as part of the routine. In intraperitoneal vaccination, the needle passed through a portion of the abdominal wall other than that where living pneumococci were subsequently implanted, so that it seems unlikely that seepage of vaccine accounts for the presence of these cells. In addition, intentional deposition of vaccine locally did not produce this picture.

After infection of most of the animals, no significant gross lesion was seen. In some mice at 24 hours and later, there were one or two firm, sharply circumscribed, yellow-gray, opaque nodules 2 mm. or less in diameter. These disappeared within 5 days. Of the mice in the survival group, eighteen of twenty lived. One died at 36 hours with a gross lesion suggesting a cellulitis.

What was the fate of living pneumococci in the subcutis containing the mononuclear cells described? As early as 5 minutes after inoculation, many of the

bacteria were within these phagocytic cells (Fig. 4). That this prompt phagocytosis was not complete or did not result in immediate destruction of the organisms was shown by the persistence of well stained, Gram-positive, intracellular cocci for several hours. It is of particular significance, however, that of all the mice examined, those of this group showed least exudation, edema and necrosis. Comparatively few granulocytes appeared in the subcutaneous tissues, and those present did not ingest the bacteria in significant numbers. The time of onset of exudation differed little from that in the control group described. Proliferative changes were slight, and did not appear until late stages, when in a few cases tiny abscesses had developed in the subcutis. Other layers of the abdominal wall were little changed. Fibrin was absent except in the abscesses.

(b) *Group Passively Immunized*.—During the first 9 hours after infection, only hyperemia of the abdominal wall was seen in the gross. Between the 9th and 24th hours, there developed in most of the mice one to three firm, yellow-gray nodules from less than 1 to 3 mm. in diameter, within the site of the bleb raised by inoculation. After the 48th hour these nodules decreased in size and disappeared. Of sixteen mice injected for survival tests, one died.

Microscopically, sections taken at 5 minutes and later disclosed sharp localization of the cocci at the site of injection, with formation of extracellular clumps of organisms similar to those pictured (Fig. 2) following simultaneous introduction into normal animals of pneumococci in saline and immune serum (see below). Phagocytosis of the clumped organisms by both mononuclear forms and granulocytes began within an hour after infection. In the earliest stages, mononuclear cells in the subcutis were slightly increased. An infiltrate composed of polymorphonuclear leukocytes, lymphocytes and macrophages appeared within an hour and progressively increased. After the 9th to 15th hours, sharply circumscribed abscesses developed in the subcutis (Fig. 6). Edema, exudation and necrosis, while not prominent, were more extensive than after intraperitoneal vaccination. Fibrin was seen only in the abscesses.

The lesions in control animals, infected after receiving normal rabbit serum intraperitoneally, did not differ in histologic details from those which followed infection of the normal mice of group 1 above.

(c) *Group Intraperitoneally and Locally Vaccinated (Immune and Hyperergic)*.—At the site of infection in the majority of the mice, developing within 24 hours, enlarging, and persisting up to 120 hours, there was a region, visible in the gross, of hyperemia and edematous thickening of the abdominal wall, averaging about 1 cm. in maximum diameter. In the remaining animals there was less edema. From 48 to 120 hours, some mice had abscesses up to 3 mm. in diameter. Of 20 mice injected for survival tests, 18 lived.

The microscopic picture in the 1st hour differed from that following infection after intraperitoneal vaccination (2 a) only in the presence of more granulocytes in the subcutis. In subsequent hours, subcutaneous edema was definitely more extensive, and infiltration chiefly by granulocytes occurred earlier and was more widespread, with greater tissue necrosis, than after intraperitoneal vaccination.

alone. Although numerous polymorphonuclear leukocytes were present in the subcutis 2 hours after infection, these cells failed to ingest significant numbers of bacteria; most of the cocci were already within mononuclear phagocytes (Fig. 9). At later stages the small abscesses seen in the gross appeared in some animals, but in others the granulocytes were suspended in edema fluid in the subcutis and not so sharply localized.

3. *Effects of an Acacia Medium.*—

(a) *Results with Acacia Alone.*—There were no significant gross changes for 9 hours after infection. The mouse killed at 15 hours was moribund; the abdominal wall was 3 mm. thick, deep red and firm in an area 2 to 3 cm. in diameter. Most of the mice killed later appeared quite sick, with increasingly wide zones of thickening of the abdominal wall, which was deep red, firm, and at late stages wrinkled, with scattered purple-red patches. As compared to changes in respective members of the control group first described, the lesions were definitely more extensive and severe, and the animals more sick. All died, mostly within 24 hours, in the survival tests. None survived for section at 72 hours.

The appearance of cellular infiltration in the subcutis was definitely delayed, with only scattered cells for 3 hours. At 4 hours, however, the exudate equalled that with saline as the medium, and thereafter surpassed it. At 12 hours the infiltrate, composed chiefly of polymorphonuclear leukocytes, was present in the subcutis, muscle layers, dermis and at the parietal peritoneum. There were many perivascular accumulations of erythrocytes. Edema was extensive and necrosis widespread, involving many cells of the exudate. The organisms, at first mostly singly and in pairs, were for the most part in small groups or clumps, often with skein formation, 1 hour after inoculation (Fig. 5). Thereafter they became disseminated through the tissue spaces. Phagocytosis was slight. 18 and more hours after infection, innumerable cocci were scattered in all layers of the abdominal wall, chiefly, however, in the deeper portions of the greatly thickened subcutis. At the 12 and 15 hour stages, there was fibrin in the subcutis; at later stages, it was present in all layers of the abdominal wall, and a thick fibrinous network developed in large areas of the subcutis and in many of the subcutaneous lymphatics.

(b) *Effects of Acacia Medium Containing Immune Serum.*—Gross lesions were insignificant for 36 hours. In most cases by 48 hours, a firm, convex, sharply circumscribed, yellow-white nodule 3 to 4 mm. in diameter occupied the center of the injection site. This lesion persisted for 6 to 8 days after inoculation. Of nine mice injected for survival tests, two died 72 hours after infection, with moderate enlargement of the gross lesion in the hours preceding death.

For 2 hours very few cells entered the subcutis. The cocci remained at the place inoculated, singly and in pairs and small clumps (Fig. 3); skein formation occurred at 1 hour. Between the 3rd and 4th hours after infection, however, the proliferating organisms became agglomerated into large clumps; these were surrounded and penetrated by polymorphonuclear and fewer mononuclear cells, with extensive phagocytosis by cells of both types (Fig. 8). In the following hours, the amount of edema and number of granulocytes, lymphocytes and large mono-

nuclear cells in the subcutis were moderately increased. At 24 hours and later, sharply circumscribed small abscesses were present in the subcutis (Fig. 7), with peripheral proliferation of mononuclear cells and fibroblasts. 144 hours after infection the proliferative changes involved small round cells in the adipose tissue, between the muscle layers and subperitoneally.

(c) *Control Group; Saline and Immune Serum.*—The gross changes in this group were not uniform. While in a few animals firm, yellow-white nodules 1 mm. and less in diameter appeared 24 hours after infection, many had lesions 2 to 3 cm. in diameter, with varying degrees of induration. Of nine mice injected for survival tests, seven died.

From 5 minutes to 1 hour after infection, the bacteria remained sharply localized in large clumps (Fig. 2). From 3 hours on, there was phagocytosis by cells, chiefly granulocytes, accumulated focally at the injection site; with increasing proliferation, however, the cocci disseminated through the tissue spaces extracellularly. In only a few instances was there a fairly well demarcated subcutaneous lesion with tendency to abscess formation. In the remaining mice, however, significant exudate was largely confined to the subcutis and adjacent borders of the dermis and muscle.

(d) *Effects of Acacia Medium after Local Active Immunization.*—Subcutaneous vaccinations led to the appearance of 3 to 6 mm. in diameter, firm, elevated, yellow-white lesions surrounded by hyperemic margins, the larger with later vaccinations. Many of these lesions became covered by crusts. Most gradually disappeared in the month following vaccination, although in a few cases, one or two tiny nodules were still visible in the gross at the time of infection. The inoculum was placed in a grossly unchanged portion of the abdominal wall.

Within 9 hours after infection, there appeared slight hyperemia and thickening of the abdominal wall. At 12 to 18 hours, the mice had indurated lesions 1.0 to 1.5 cm. in diameter. At 24 hours, each showed a firm, reddened, thickened region 1.5 to 4.0 cm. in diameter, averaging about 2.5 cm. By 72 hours, most mice had died with the entire abdominal wall deep red, thick, wrinkled and studded with hemorrhages; in a few cases the lesions remained only 1 cm. in diameter. Within 120 hours all the mice had died.

A detailed description of the histologic changes is not necessary. The lesions were similar to but even more extensive and destructive than those of normal mice receiving pneumococci in acacia alone (3a). Peritonitis developed within 15 hours. Proliferation of the cocci in late stages was extreme (Fig. 12).

(e) *Control Group; Pneumococci in Saline after Local Vaccination.*—For 9 hours after infection, the changes were similar in the gross to those in mice of group 3d. Up to 24 hours, most mice had a soft, pink-white, slightly elevated lesion about 1 cm. in diameter. Thereafter these became deeper red, firmer and slightly more extensive. At 48 hours they were similar. By 72 hours after infection, about half the animals exhibited lesions double this size, red, thick and firm, and these mice died; in the rest, the lesion became progressively smaller, with formation of round, firm, yellow-white, 2 to 4 mm. nodules.

In the presence of immunization obviously inadequate for complete protection, the histologic picture was as varied as the gross. The exudate, at first patchy, became more diffuse as cocci spread from the sites at which they were more or less localized during the first 3 hours. In the subcutis especially, edema and necrosis were often extensive. In the presence of exudate spread in the later stages throughout the dermis, subcutis, muscle and to the peritoneum, however, comparatively few cocci were found.

COMMENT

If intraperitoneal immunization is essentially similar to intravenous, our observations on the reactions of immunized mice resemble those of Swift and his associates (16), who described the types of response of immunized rabbits to intracutaneous infection with non-hemolytic streptococci. These investigators reported that intravenous vaccination led to the production of a small, hard, shotty lesion on subsequent intracutaneous infection; this type of reaction was regarded as "immune" or "hypoergic," and provided adequate protection. Repeated, local inoculations of a small amount of vaccine favored the appearance of a much more extensive, soft lesion with increased edema, exudation and necrosis,—a "hyperergic" reaction. Since allergy is literally merely altered reaction, the above distinction between hypoergic and hyperergic types of allergy seems appropriate. An animal may be protected in the presence of either reaction, depending on the technique of immunization. Rich minimized the importance of the hyperergic response in localization of pneumococci in rabbits. After studying the specific response of rabbits resistant or hypersensitive following administration of streptococcus protein, Clawson (17) concluded that hypersensitiveness when present is merely a harmful concomitant phenomenon. Cannon and Pacheco from their observations on guinea pigs felt that the hyperergic (as they termed it "anaphylactic") inflammation was of importance in promoting continued bacterial localization; intravenous or intraperitoneal immunization was not used in their work. Since the hyperergic reaction at least results in increased local tissue destruction, and perhaps an accompanying more severe intoxication, and since this more violent reaction may actually favor dissemination of the infecting organisms, protection secured without such a reaction seems significant. Under our experimental conditions, such protection was afforded by intraperitoneal immuniza-

tion. When a hyperergic response was induced by additional, local vaccinations, the many granulocytes of the exudate apparently had little or no part in phagocytosis.

In passively immunized animals, and in those receiving an inoculum containing immune serum, we observed agglutination *in vivo* as described by Tsuda (18), Rich, and Cannon and Pacheco. This phenomenon probably results from reaction between type specific antibody and capsular polysaccharide, with alteration of bacterial surface properties, so that the organisms become susceptible to agglomerating forces which promote localization, and to phagocytosis. In the case of pneumococcal infection in an animal normally susceptible, there is little evidence that the specific humoral factor is alone adequate for disposal of the bacteria, without participation by host cells. Tsuda, working with streptococci and pneumococci in mice, reported the organisms were destroyed in the tissue fluids in the absence of cellular reaction after a high degree of immunity had been induced. Goodner and Stillman (19), in evaluating active resistance of rabbits to pneumococcal infection, found that a group responding with no lesion whatever was largely hypothetical. Only a few such instances were seen over a period of years, and in these, the animals had received many courses of intravenous vaccination with heat-killed pneumococci. While Rich at first thought the agglutination alone did not maintain localization of *P. aviseptica* and pneumococci in rabbits. When the possibility of a leukocytic response was removed by benzolization, the bacteria ultimately disseminated from the site of injection and caused death of the host. In our experiments, agglutination, occurring within 5 minutes of infection, assured localization of the bacteria for a period during which they continued to proliferate. The ultimate dissemination occurring in the presence of a subeffective amount of immune serum, even with extensive cellular infiltration, demonstrated the necessity of an adequate concentration of immune bodies if the organisms were to be ingested. But even with sufficient antibody, the organisms were never eradicated without cellular participation. The chief importance of agglutination *in vivo* seems then to be as an early localizing mechanism.

In the presence of specifically mobilized macrophages, agglutination

was not observed. Instead, phagocytosis began almost immediately, and in a short time very few cocci could be seen extracellularly. In these cases the organisms apparently adhered to the tissues at the site of injection and to phagocytic cells rather than to each other. Phagocytosis well advanced within the early minutes of infection precluded the possibility of formation of clumps of proliferating cocci.

No exact determination of the origin of these phagocytic cells was attempted. Of possible significance are the numerous circulating lymphoid cells normally possessed by the mouse (21), and our observations of diapedesis of such cells in all stages of exudation following infection. Transitional forms between granulocytes or fibroblasts and mononuclear phagocytic cells were not seen.

We have no evidence that a mechanical barrier of fibrin in the tissue spaces and lymphatics is of importance in localization of virulent pneumococci by the mouse. In a series of reports Menkin (22) has emphasized the importance of this barrier in the localization of bacteria and other particulate material in a field of inflammation. Of interest in this connection is Goodner's (23) recent observation that repeated addition of thromboplastin to the lesion of dermal pneumococcus infection in the rabbit delayed spread of the lesion for 2 hours after the last addition. Others (Rich, Rhoads and Goodner, Pacheco (24)) have either seen no fibrin in the tissues during the first 48 hours of acute inflammation or have attributed bacterial localization to other factors. In the lymphatics draining a region of sterile inflammation in the dog's paw, Field, Drinker and White (25) found the lymph pressure and flow markedly increased for at least 8 hours. In studies of lymphatic drainage from similar lesions in rabbits, Menkin (26) found no retention of trypan blue in the lesions for the first 18 hours. He earlier reported that this dye was localized in a field of pneumococcal inflammation rather promptly, but his table shows that the dye did not fail to drain into the lymphatics when introduced up to 17.5 hours after infection (27). In our experiments, pneumococci were localized by forces acting much earlier after infection. Significant amounts of a fibrinous network in the tissues and of thrombosis of lymphatics were seen in precisely those lesions which accompanied failure to localize the pneumococci. Perhaps under other conditions, as when a less virulent organism can be localized in the absence of previous immunization, a barrier of fibrin may play a significant rôle.

An acacia medium may promote bacterial agglomeration *in vitro*. Moretti and Aragona (28) reported progressively increasing agglomeration of a variety of organisms with increasing concentrations of solution of acacia from 2 to 10 per cent. Went (29) found the sensitivity of a number of organisms to agglomeration by acacia correlated with the surface area. Ciliated forms flocculated at a low viscosity; staphylococci did not at all, even after 8 hours in an acacia medium with a relative viscosity of 60.

Reports of the effects of acacia on immunagglutination are not in complete agreement. Went found that increasingly viscous solutions of acacia delayed immunagglutination *in vitro* through hindrance of adsorption of antibody by the bacteria. Reimann's work demonstrating enhanced agglutination, notably at viscosities 3.5. to 5.6 times that of water, has been mentioned. In our own *in vitro* work, the optimum viscosity was essentially similar. For example, agglutination occurred to 1:40 in saline, increased with higher concentrations of acacia to 1:2560 in 5 and 6 per cent, and sharply decreased to 0 in 10 per cent; there was no agglomeration in the absence of immune serum. Agglutination at the higher dilutions of antibody was present 2 hours after admixture and was increased after the tubes remained in a refrigerator overnight. Although solution of acacia may at first delay immunagglutination, we have found the latter ultimately enhanced in certain concentrations of this medium.

We have repeated Reimann's intraperitoneal injections of pneumococci into mice, comparing the effects of saline and acacia media with and without immune (and also normal) serum, and have had essentially similar results. We did, however, obtain much more phagocytosis than was reported by Reimann when acacia containing immune serum was used (Fig. 10).

The effect of a 10 per cent acacia medium in the tissues of the abdominal wall was to hinder bacterial dissemination in the early stages. The clumps and skeins prominent at the 1 hour stage probably were made up of cocci that failed to disseminate through the viscous medium as they proliferated, rather than of organisms agglomerated after first being scattered. Ultimate dissemination apparently accompanied dilution of the acacia. The intense exudation and widespread necrosis might be partly explained by the irritating effect of the acacia itself, especially in the crude form used; more

important, a protective coating may have been formed on the bacteria. Nungester, Wolf and Jourdonais (30) reported such protection afforded Type II pneumococci, hemolytic streptococci and staphylococci by 4 per cent gastric mucin and other viscous substances in the peritoneal cavity of the mouse, though not in the subcutis. The early hindrance to ingress of host cells does not seem so important, because these cells did not take up significant numbers of pneumococci introduced in a saline medium. Actual clumping of the host cells, as in the peritoneal cavity, did not occur in the subcutis.

In definite contrast were the results of injecting pneumococci in acacia containing a small amount of immune serum. Prompt agglutination as with the saline medium was prevented, and the early free ingress of phagocytes also. But within a few hours large clumps of bacteria were surrounded and invaded by actively phagocytic cells, at a stage when the amount of immune serum used no longer maintained localization in the absence of acacia. Thus free diffusion of antibody, its adsorption on the bacteria and agglutination were apparently delayed, but the localizing effects of acacia were sufficient to prevent dissemination of the bacteria in the earliest stages. At later periods the combined effects of acacia and antibody favored formation of a small, sharply circumscribed abscess and protection of most of the mice.

These results with a small amount of immune serum practically rule out the existence in acacia of a carbohydrate exerting an aggressin-like action through its close chemical similarity or identity to the Type I pneumococcus polysaccharide. Such a "soluble specific substance," having a high precipitating activity with Type II and Type III anti-pneumococcus sera but not Type I, has been derived from gum acacia by partial acid hydrolysis (31).

After local active immunization, presumably leading to formation of immune bodies in the tissues, results were not clear-cut. The degree of immunization was inadequate, or the length of time before infection too long, to afford complete protection when a saline suspension of organisms was injected. The extent of the inflammatory reaction was probably partly due to a hyperergic reaction. The severe lesions with the bacteria in acacia, however, and the death of all

animals so inoculated suggest that antibody penetrates from the tissues into such an inoculum with difficulty. In support of this, mice injected intraperitoneally with 0.01 cc. of immune serum before subcutaneous inoculation of living pneumococci were protected when a saline medium was used, while less than half survived after introduction of an acacia suspension of the organisms.

SUMMARY

The results of gross and microscopic observations and survival tests after subcutaneous infection of mice with Type I pneumococci can be summarized as follows:

Normal mice possessed no adequate means of protection against this organism. The bacteria disseminated freely through the tissue spaces in the presence of widespread inflammation, and were not ingested by significant numbers of host cells.

Macrophages mobilized in the subcutis by intraperitoneal vaccinations with homologous killed organisms promptly ingested the living bacteria subsequently introduced. The cocci were sharply localized and destroyed in the presence of minimal amounts of exudation, edema and necrosis. Bacterial localization accompanied a similar hypoergic reaction after passive immunization. When a hyperergic response was induced by repeated local, in addition to intraperitoneal, vaccinations, prompt phagocytosis by macrophages, as after intraperitoneal vaccination alone, was again the outstanding means of disposal of the organisms. The heightened exudation, edema and local necrosis resembled a harmful rather than an important localizing reaction. Immunity was not dependent upon a hyperergic reaction.

Prompt *in vivo* agglutination of living bacteria after passive immunization was an important early localizing phenomenon. After either active or passive immunization, phagocytosis was the only means observed by which the organisms were destroyed. Thus localization and destruction of the bacteria in immunized mice were dependent both on bacterial changes caused by specific antibody and on phagocytic activities of host cells.

A viscous gum acacia medium caused a transient delay in bacterial dissemination, but the organisms ultimately became widely scattered

in the tissues of the abdominal wall. The animals died earlier, with more extensive and destructive lesions than when the bacteria were introduced in a saline medium.

In distinct contrast, the pneumococci were as a rule sharply localized and destroyed when injected in an acacia medium containing a small amount of immune serum.

After local active immunization, sufficient to protect about half the animals subsequently infected by pneumococci in a saline medium, extensive and destructive lesions and death of the mice followed introduction of the organisms in acacia. It was suggested that the more viscous medium may have hindered diffusion of immune bodies from the tissues into the inoculum.

Sincere thanks are extended Dr. Paul R. Cannon for his many valuable suggestions.

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EXPLANATION OF PLATES

PLATE 35

FIG. 1. Pneumococci scattered extracellularly in the subcutis 5 minutes after injection into a normal mouse. Brown and Brenn stain. $\times 1175$.

FIG. 2. Subcutis of normal mouse 5 minutes after injection of a saline suspension of pneumococci and 0.001 cc. of immune serum. Note the prompt and definite agglutination of the bacteria. Brown and Brenn stain. $\times 1175$.

FIG. 3. Slight clumping of the bacteria 5 minutes after they were introduced in an acacia medium containing 0.001 cc. of immune serum. Brown and Brenn stain. $\times 1175$.

FIG. 4. Prompt ingestion of pneumococci by mononuclear phagocytes. Mouse killed 5 minutes after infection following intraperitoneal vaccinations. Brown and Brenn stain. $\times 1175$.

FIG. 5. Formation of small clumps and skeins in the subcutis 1 hour after injection into normal mouse of pneumococci suspended in solution of acacia. Brown and Brenn stain. $\times 1175$.

PLATE 36

FIG. 6. Tangential section showing sharply localized abscess 24 hours after infection following passive immunization (serial sections show that the abscess is in the subcutis). Maximow stain. $\times 35$.

FIG. 7. Tangential section showing subcutaneous abscess 48 hours after injection of an acacia suspension of the pneumococci and 0.001 cc. of immune serum. Maximow stain. $\times 35$.

FIG. 8. Penetration of large bacterial clumps by phagocytic cells 4 hours after introduction of the cocci in acacia with immune serum. Maximow stain. $\times 775$.

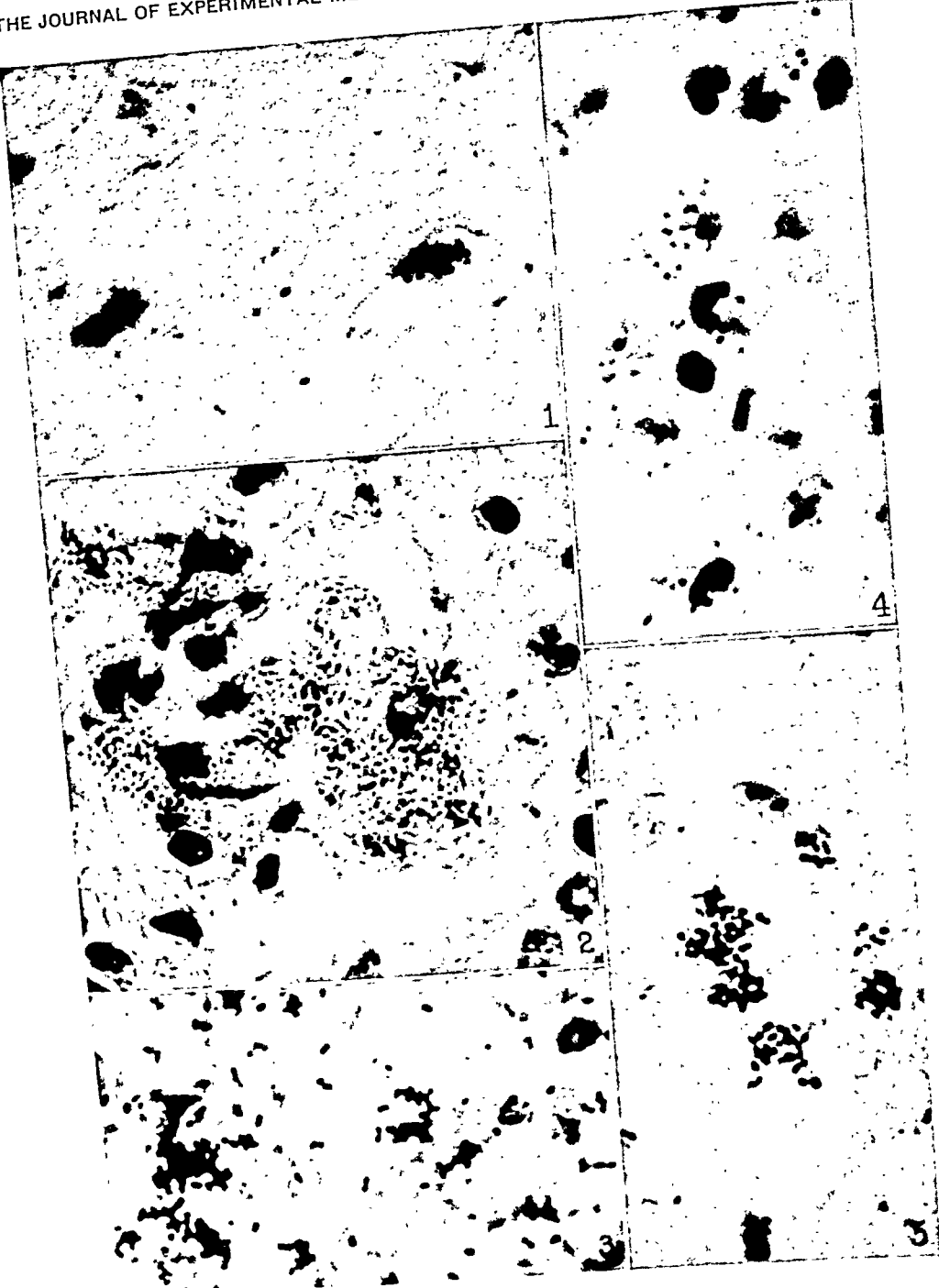
FIG. 9. Subcutis of immune and hyperergic animal 2 hours after infection. Most of the cocci are in mononuclear phagocytes; the part played in phagocytosis by granulocytes is negligible. Brown and Brenn stain. $\times 975$.

FIG. 10. Phagocytosis by macrophages at periphery of large clump of cells and bacteria. Smear of peritoneal fluid 30 minutes after intraperitoneal injection of pneumococci in salt followed by 10 per cent acacia and 0.01 cc. of immune serum. Giemsa stain. $\times 1450$.

PLATE 37

FIG. 11. Nest of mononuclear phagocytic cells in the subcutis after repeated intraperitoneal vaccinations. Maximow stain. $\times 500$.

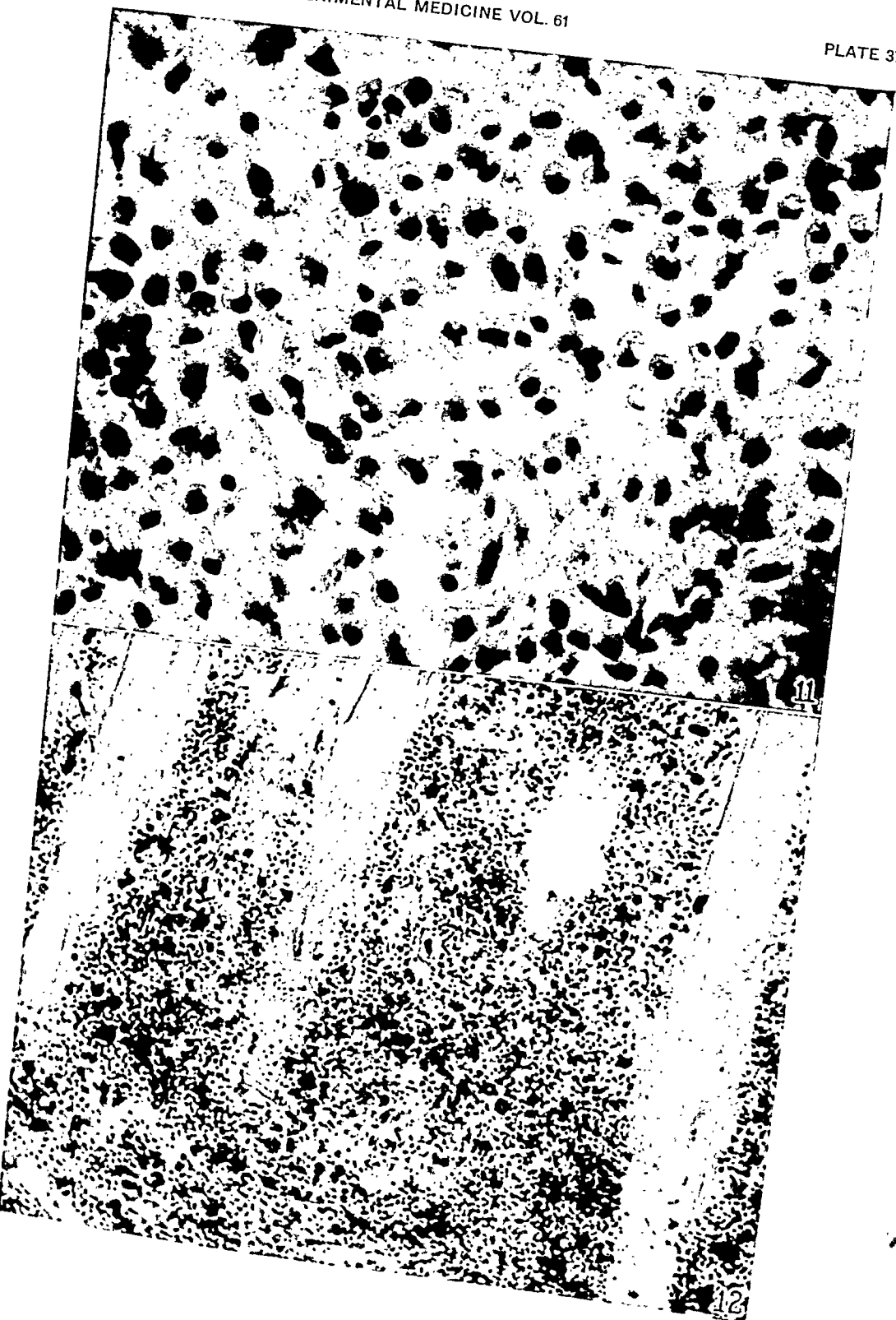
FIG. 12. Extreme bacterial proliferation and invasion from the subcutis into the muscularis 36 hours after infection with acacia-suspended bacteria in mouse locally vaccinated. Brown and Brenn stain. $\times 550$.



(Catron: Bacterial Localization)



(Caption: Bacterial Localization)



(Left = External location)

STUDIES ON MENINGOCOCCUS INFECTION

VIII. THE TYPE I SPECIFIC SUBSTANCE

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In a previous paper (1) the preparation of fractions from the meningococcus with marked type specificity toward antimeningococcal monovalent serum was described. For the most part these fractions were obtained from autolysates of the meningococci growing in hormone broth. They were not pure chemical entities and, besides being rarely able to evoke the precipitin reaction in dilutions higher than 1:10,000, gave cross-reactions with heterologous serum. An additional fact noted was that, as far as the type-specific substances were concerned, Types I and III were serologically identical. Subsequently (2) a method was described briefly for the preparation of a type-specific substance from Type I meningococcus which was pure and highly active serologically. In the present communication a more detailed account of the preparation and properties of this substance, to be referred to as S I, will be given.

Material and Technique of Preparation

Strains of Type I meningococci were obtained from sources previously acknowledged (3). For the most part they were used shortly after isolation, that is within 10 weeks, though in one case a strain was used after it had been maintained on artificial media for more than 12 months. In this case the yield of type-specific substance was poor, and in general it has been found that the growth characteristics of the strains might become unsatisfactory in a very short period. Thus Strain 503, which gave a good yield of S I on Apr. 23, 1934, (preparation 19), being then 23 days old, gave only a small yield when inoculated on May 4 and again on May 10, 1934. This loss in the power to produce the type-specific substance is in keeping with the observations of Petrie (4) and the present authors (3). Some strains, as for example 502, continued to give an excellent yield after 12 weeks but in general the sooner the strain was utilized after isolation the better the results.

For the preparation of S I, autolyzed cultures were found most convenient. This fact has been mentioned before (1, 5) and it is only necessary to repeat here that the S I can be obtained by the various methods of extracting young, active cultures washed off solid media. However, these methods are more time-consuming and give a much smaller yield and for these reasons autolysis in broth has been the method adopted.

The organisms were grown in 1 liter or 2 liter Florence flasks containing 600 cc. or 1200 cc. of hormone broth, respectively, at pH 7.0 to 7.2 and containing 0.3 per cent glucose. The glucose increased the amount of growth and it was found that the substitution of this pH for the usual one of 7.6 caused growth to begin more readily and proceed more rapidly. The inoculum was 5 to 10 cc. of an actively growing culture in hormone broth. The flasks were capped with lead foil and incubated at 37°C. After pellicle formation began they were agitated daily to break this pellicle and allow new growth to occur at the surface. Growth ceased within 10 to 18 days. Each flask was then examined for the presence of contaminants in stained smears and if these were found, that flask was discarded.

Isolation of the Type-Specific Substance

The fact has already been noted (1, 2) that the protein precipitable from Type I meningococcus autolysates by dilute acetic acid, the so called nucleoprotein P fraction, carries with it appreciable amounts of S I. This reaction has been utilized as the first step in isolating purified S I. It was first observed that when the P fraction was precipitated by acidifying the broth to pH 4.0 with acetic acid in the presence of 1.5 volumes of ethyl alcohol, the S I present was removed almost quantitatively from solution (as shown by the precipitin test with Type I monovalent antimeningococcal serum¹). Since the large

¹ The precipitin test now used shows slight modifications from the method previously described (1). Tubes of about 3.5 mm. internal diameter are used. The serum is introduced first and the solution carefully layered on top. The difference lies in the fact that the overnight reading is usually omitted and the immediate, 1 hour, and 2 hour tests are all made by the ring method; *i.e.*, without mixing the two fluids. The use of tubes of smaller diameter facilitates this, as appreciable mixing of the fluids does not occur even after 2 hours at 37°C. provided agitation of the tubes be carefully avoided. More sensitive readings can be obtained by this method. It is, however, unsuitable for comparative estimation of the amount of precipitate which occurs, and for this the method previously described is still used. It should be emphasized that the use of a strong beam of light and a dull black background against which to observe the tubes not only facilitates all readings but is essential for reading finer degrees of precipitin reaction.

scale precipitation of unconcentrated broth with ethyl alcohol was not practical, experiments were carried out starting with broth which had been concentrated *in vacuo* to 1/10 or 1/15 the original volume. When this material was acidified to pH 4.0 with acetic or hydrochloric acid in the presence of as much as 1.75 volumes of ethyl alcohol, only about one-half of the S I appeared in the precipitate. The use of larger amounts of alcohol was precluded because this caused the constituents of the broth to precipitate out as a gum.

Since concentration of the broth before precipitation rendered the adsorption of the S I on the P fraction less efficient than when unconcentrated broth was used, it seemed possible that preliminary dilution of the broth might prove more satisfactory and it was soon shown that the adsorption could be made nearly quantitative (as shown by the precipitin test), without the use of ethyl alcohol, if the broth was diluted with water before acidification.

The optimal dilution was found to vary from 1:1 to 1:4 with different batches of broth. It should, ideally, be determined for each preparation. However, the increased efficiency of the adsorption at higher dilutions is offset by the more cumbersome manipulation entailed and by the presence of serologically inactive polysaccharides in the final product, the proportion of which increases with increasing dilution. In one preparation in which the broth autolysate was diluted with 9 volumes of water before acidification, the inactive polysaccharides formed the bulk of the product.

These considerations led to the adoption of the following procedure which proves satisfactory with 18 liter quantities of broth.

The autolysate is divided equally between two 5 gallon bottles, diluted with an equal volume of cold tap water and allowed to stand at 4°C. overnight. The next morning it is stirred and acetic acid is slowly added in the proportion of 10 cc. of acid per liter of original broth autolysate. The resultant pH, usually 4.0, is equal to or more acid than the isoelectric point of the P fraction which begins to flocculate out. The bottles are returned to the refrigerator for 24 hours² when the bulk of the precipitate is found to have settled to a fairly compact layer several centimeters in thickness. The clear or slightly cloudy supernatant is siphoned off as completely as possible and the precipitate collected by centrifugal-

² The S I is sensitive even to weak acid if the temperature is sufficiently high. Hence, as a matter of precaution, solutions were chilled whenever it was necessary to expose the preparation to acid conditions for more than a few minutes.

ization, preferably in the cold.³ The precipitate is then homogenized in 1 liter of distilled water very slightly acidulated with acetic acid and the suspension filtered on hard filter paper sheets 38 cm. in diameter in the refrigerator. The filtration is complete in 24 to 48 hours when the filtrate is discarded since it contains little or no S I.

Having determined the optimal conditions for the primary adsorption, the problem remains to separate the S I from the relatively enormous amounts of protein which accompany it (20 to 100 times the amount of S I) and to which it seemed to be firmly attached. The combination proved, however, to be a loose one and it was possible to dissociate the complex and remove about 90 per cent of the protein by reprecipitation at pH 4.8 to 5.0 in the following manner. The precipitate of crude P fraction is readily scraped off the filter paper and is homogenized in a volume of distilled water not greater than 900 cc. in an Erlenmeyer flask. Normal sodium hydroxide is added slowly with stirring until pH 7.0 is reached and the stirring is continued until all lumps disappear. 10 gm. of sodium acetate are then added (in the absence of this electrolyte reprecipitation of the P fraction at pH 4.8 or 5.0 does not occur readily) and dissolved, followed by the calculated amount of normal acetic acid (for 10 gm. of sodium acetate [Green (6)]) necessary to produce pH 5.0, namely 35 cc. After the solution has stood for 10 to 15 minutes the precipitate may begin to separate leaving a narrow ring of clear fluid at the top. If this does not happen, 5 cc. portions of normal acetic acid are added with stirring until precipitation does occur. The amount of acid needed varies from preparation to preparation and indeed is not critical since there is little danger of making the solution too acid by using the procedure described. The precipitation mixture is filtered on hard paper in the refrigerator for 24 to 48 hours. The clear, slightly yellow filtrate (pH 4.8 to 5.0; *gamma* dinitrophenol indicator) is neutralized with normal sodium hydroxide and set aside in the refrigerator with chloroform as a preservative. The precipitate is scraped off the filter paper and subjected once more to the process outlined above of solution followed by reprecipitation at pH 4.8 to 5.0. This repetition usually calls for more acetic acid than at first. The mixture is now filtered on soft paper in the refrigerator and at the end of 24 to 48 hours transferred to a large Buchner funnel and sucked dry. It is possible to dissociate a small additional amount of S I from the P fraction by a third solution and reprecipitation, but this is usually not profitable. The combined neutralized filtrates contain the bulk of the S I accompanied by a very considerable amount of protein, colored material and sodium acetate. They are concentrated *in vacuo* to a volume of 150 to 200 cc. The S I and protein are precipitated by the addition of 5 volumes of ethyl alcohol, the mixture standing overnight in the refrigerator. The precipitate is centrifuged

³ This, and the succeeding precipitates of the P fraction, are bulky, gelatinous and do not pack well when centrifuged. For this reason, filtration has been the method generally chosen for collection of this material. It is a slower technique but is less laborious and gives more nearly complete separation.

off and the supernatant, which contains much pigment and most of the sodium acetate, is discarded. The precipitate is dissolved in 150 to 200 cc. of distilled water and concentrated *in vacuo* to a volume of 50 to 70 cc. in order to remove the alcohol. Most of the remaining protein is now precipitated by adding the calculated amount of ammonium sulfate necessary to produce a 0.7 saturated solution; namely, 0.5 gm. per cc. of solution.⁴ The precipitate, which contains very little S I, is collected on soft paper in a Buchner funnel and discarded. For removal of the ammonium sulfate the clear, light yellow filtrate is neutralized with sodium hydroxide and dialyzed through cellophane⁵ in a bath of running tap water until it gives only a slight test for the sulfate ion. Chloroform is added as a preservative. It is usually necessary to concentrate the solution *in vacuo* once during the course of the dialysis. The last of the sulfate is removed by adding 10 per cent barium acetate solution to the dialysate until further increments produce no additional precipitate. The excess barium is removed by adding enough sodium carbonate to make a 5 per cent solution and the precipitate of barium carbonate and barium sulfate is centrifuged off. The clear, often colorless supernatant is then acidified with acetic acid to pH 4.5, concentrated *in vacuo* to 40 cc. and transferred to a 250 cc. centrifuge bottle. The S I is precipitated by adding 4 volumes of ethyl alcohol and centrifuged off after standing in the refrigerator 5 or 6 hours or preferably overnight. In the presence of an excess of sodium acetate 4 volumes of ethyl alcohol precipitate the S I almost quantitatively. On centrifuging, the S I forms a compact button of yellowish gum. This material is pure enough for serological purposes and if desired may be rinsed off with ethyl alcohol and dried in a vacuum desiccator.

For further purification the gum is dissolved in 20 cc. of 5 per cent copper acetate solution. The resultant precipitate, usually small in amount, contains the remaining protein and most of the pigment. It is centrifuged off at high speed leaving a clear or slightly opalescent supernatant. To this are added 10 cc. of distilled water, 2 gm. of sodium acetate and 3.5 cc. of acetic acid which ensure the copper remaining in solution when the S I is precipitated with 4 volumes of ethyl alcohol. The S I is dissolved in 3 or 4 cc. of distilled water and if there is much

⁴ Since these experiments were completed, it has been found that copper acetate solution, buffered at pH 5.0 or slightly higher (just yellow to methyl red indicator) by the addition of sodium hydroxide, is much more satisfactory and efficient than is ammonium sulfate for the removal of protein at this stage of the preparation. The copper acetate is removed by repeated precipitation of the S I with 4 volumes of ethyl alcohol from a solution containing 10 per cent sodium acetate and sufficient acetic acid to maintain pH 4.0. Optimal conditions for this technique have not been completely determined, so that details of procedure cannot be given at this time.

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For further purification the gum is dissolved in 20 cc. of 5 per cent copper acetate solution. The resultant precipitate, usually small in amount, contains the remaining protein and most of the pigment. It is centrifuged off at high speed leaving a clear or slightly opalescent supernatant. To this are added 10 cc. of distilled water, 2 gm. of sodium acetate and 3.5 cc. of acetic acid which ensure the copper remaining in solution when the S I is precipitated with 4 volumes of ethyl alcohol. The S I is dissolved in 3 or 4 cc. of distilled water and if there is much

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insoluble matter the copper acetate treatment is repeated. The S I can be freed of all traces of copper by two or more precipitations from acid solution as follows: The S I is dissolved in 40 cc. of 10 per cent aqueous sodium acetate and freed of insoluble matter by centrifuging; 6 cc. of acetic acid is added and precipitation is brought about by the addition of 4 volumes of ethyl alcohol. The product now contains S I and varying amounts of a serologically inactive polysaccharide. The latter has usually been present in only small amounts and can be removed by dissolving the S I in not more than 10 cc. of 20 per cent aqueous sodium acetate, chilling the solution in an ice bath and adding 4 volumes of cold acetic acid. After 1 to 2 hours at 0°C. the inactive material appears as a small amount of gelatinous precipitate which is removed either by centrifuging or filtering in the cold.

In certain cases, notably those in which the amount of S I in the original broth was scant and in one case where the broth was originally diluted tenfold, 50 per cent or more of the product at this stage consisted of this inactive fraction. Its presence in large amounts was shown by the fact that the product, separated out from the sodium acetate solution by means of ethyl alcohol, appeared almost immediately as a flocculent precipitate, whereas under these conditions the purified S I always separated out slowly (6 hours or overnight at 0-4°C.) as a gum. When present in such large amounts complete separation of this inactive fraction from the S I was not possible. Fairly good results might be obtained by the acetic acid precipitation outlined above, using, however, 40 cc. of 10 per cent sodium acetate as solvent and 4 to 10 volumes of acetic acid as precipitating agent. On account of the bulky gelatinous nature of the precipitated inactive fraction it was necessary to repeat the process.

The S I is now precipitated in a white, rather gelatinous form by addition of 4 volumes of ethyl alcohol to the supernatant from the precipitation of the inactive fraction with acetic acid. The precipitate forms almost immediately and is centrifuged off after 15 to 30 minutes. It is dissolved in 40 cc. of aqueous 10 per cent sodium acetate and the solution is freed of any insoluble material by centrifuging. The S I is precipitated with 4 volumes of ethyl alcohol as a colorless or slightly yellow gum and this last procedure is repeated to ensure complete removal of free acetic acid. Finally, the S I is taken up in 10 cc. of neutralized 20 per cent aqueous sodium acetate and precipitated by the addition of 200 cc. of absolute ethyl alcohol as a white, finely divided product. The precipitate is collected by centrifuging and dried in a vacuum desiccator. The yield from 18 liters of broth varies from 0.3 to 0.8 gm.

The product in the finely divided state described is unsuitable for analytical work, since it cannot be satisfactorily freed from sodium acetate by washing, owing to its tendency to form colloidal suspensions in alcohol when an excess of sodium acetate is not present. In preparing samples for analysis the following method has been used, and while this procedure is not entirely satisfactory, nevertheless, owing to

the difficulty experienced in precipitating the S I from pure solvents, it has proved to be the best method available.

A suitable amount of the S I (0.1 to 0.25 gm.) is dissolved in 5 to 10 cc. of a neutralized 10 per cent aqueous solution of sodium acetate. The solution is centrifuged at high speed and transferred to a weighed 50 cc. centrifuge tube. The S I is then precipitated in the gummy state by adding 4 volumes of redistilled ethyl alcohol and allowing the mixture to stand overnight in the refrigerator. The precipitate is centrifuged off and the tube is allowed to drain for several minutes by resting it in the inverted position on a piece of filter paper. The tube is then rinsed out with several portions of 10 to 20 cc. of redistilled ethyl alcohol allowing the gum to remain in contact with each portion for at least 2 hours. Finally, after draining it thoroughly, the tube is dried to constant weight under a high vacuum in the presence of calcium chloride and sodium hydroxide. The S I is then dissolved in distilled water and made up to such a volume as to contain 10 mg. per cc. The solutions are stored in the refrigerator with a little chloroform as preservative and portions are withdrawn as required.

Analysis

All the analytical data given represent an average of two or more determinations.

Carbon, hydrogen and micro Dumas nitrogen determinations were carried out⁶ in the customary manner, using solid samples prepared by precipitation with 20 volumes of absolute ethyl alcohol. It should be noted that the figure given for carbon is doubtless low due to the great difficulty experienced in burning the ash free of carbon. Phosphorus was determined by Elek's modification of the micro method of Lieb (7). Aliquot portions of the standard solutions were evaporated to dryness at 100°C. in the silver crucible used for digestion. For the ash determinations aliquot portions were evaporated to dryness at 100°C. in a small platinum crucible which was then ignited to constant weight. No sulfuric acid was added since the phosphorus content of the S I is more than large enough to convert all the sodium to sodium phosphate. Calculation of the sodium was made on the assumption that the ash was $\text{Na}_4\text{P}_2\text{O}_7$. Qualitative tests on the ash were carried out according to Noyes (8). Nitrogen was determined by a slight modification of the Pregl micro Kjeldahl procedure (9). Reducing sugars were determined by the Shaffer-Hartmann micro method (10) on samples which had been subjected to the following method of acid hydrolysis.

The aliquot portion (about 2 mg.) of a standard solution of S I was pipetted into the tube in which the determination was to be made; sufficient hydrochloric

⁶ The determinations were carried out by Mr. D. R. Rigakos to whom our thanks are due.

acid was added to make a final concentration of 1.0 normal; and hydrolysis was carried out at 100°C. for $\frac{1}{2}$ hour. Longer heating did not increase the yield of reducing sugars. The hydrolysate was always neutralized with normal sodium hydroxide and diluted to the proper volume before the Shaffer-Hartmann reagent was added.

The ash left after ignition of samples of S I has been identified as a sodium phosphate, probably sodium pyrophosphate. It forms a clear melt in the crucible at medium red heat, and is readily soluble in water giving a solution strongly alkaline to phenol red. No gas is evolved when the ash is treated with dilute acid. Solutions of 3 mg. of the ash give an immediate reaction with ammonium molybdate reagent. Careful analyses of 20 mg. samples have shown the absence of all cations up to the alkali group. For the positive identification of sodium, 53 mg. of preparation 17 was ashed in a platinum crucible and ignited until all the carbon was burned. The ash was dissolved in 1 cc. of distilled water and to this solution in a small test tube was added 2 cc. of potassium pyroantimonate solution (8). After several minutes the typical heavy crystalline precipitate of sodium pyroantimonate began to settle rapidly. There was no evidence of non-crystalline precipitate.

The following experiment showed that the phosphorus is firmly held in organic combination. To 2 cc. of 0.1 per cent solution of S I (2 mg.) was added 5 cc. of dilute nitric acid and 15 cc. of ammonium nitrate solution (all solutions being prepared according to the directions given by Treadwell and Hall (11)). The solution was warmed to 50° or 60°C. and 10 cc. of ammonium molybdate solution at the boiling point was added. A yellow color developed but only a slight precipitate of ammonium phosphomolybdate appeared even on standing overnight at room temperature. As a control a similar amount of S I was tested after it had been boiled for 15 minutes in 50 per cent nitric acid. The test was strongly positive as soon as the various reagents had been added.

Analytical data of several preparations are summarized in Table I. Nos. 8 and 9 were early preparations and were not subjected to either the copper acetate treatment or the precipitation with 4 volumes of acetic acid. Instead they were purified by repeated precipitation from a solution of the substance in 10 per cent aqueous sodium acetate by 4 volumes of ethyl alcohol. It is interesting to note that the properties of preparation 9, made from a stock strain, were not significantly different from the properties of the other preparations all of which were from freshly isolated strains. The yield in this case, however, was very small, being only 0.12 gm. from 40 liters of broth. If the early preparations are disregarded it will be seen that, in the last four preparations (including 15 B which was not subjected to the barium acetate and sodium carbonate treatment) made from three

different strains, a product has been obtained with a nearly constant composition and an average content of 4.36 per cent nitrogen, 8.91 per cent phosphorus and 9.59 per cent sodium. On acid hydrolysis an average of 45.2 per cent reducing sugars calculated as glucose is formed. The average optical rotation is $+56.8^\circ$ for the sodium D line.

The biuret and trichloroacetic acid tests have been negative when carried out on 10 mg. samples of S I, indicating absence of appreciable quantities of protein. Tryptic digestion does not destroy the sero-

TABLE I
Summary of Analytical Data. Type I Meningococcus Specific Substance

Preparation No.	Strain No.	C*	H*	N*	Ash as Na	P*	Reducing sugars on acid hydrolysis as glucose*	$[\alpha]_D^\circ$	Precipitin titer†
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>degrees</i>	
8	484 + 489	30.37	6.0	4.90†	10.2		46.6	$+56.8$	1:8,000,000
9	455			4.70	12.7		44.5	$+55.5$	1:8,000,000
15 B	497			4.53	9.55	8.83	44.7	$+55.9$	1:8,000,000
17	497			4.44	9.80	9.01	47.0	$+56.0$	1:8,000,000
18	502			4.20	9.74	8.93	44.3	$+57.0$	1:8,000,000
19	503			4.28	9.27	8.88	44.8	$+58.4$	1:8,000,000
Average of 15 B, 17, 18, 19....				4.36	9.59	8.91	45.2	$+56.8$	

* Ash-free.

† Vs. Type I antimeningococcal horse serum.

‡ Micro Dumas.

logical activity of the preparations. Addition of a little iodine and potassium iodide solution to a 5 mg. sample produces no red or blue coloration indicating an absence of glycogen and starch. The Molisch test has been positive in dilutions as high as 1:100,000. The orcinol-hydrochloric acid test was negative when carried out on a 5 mg. sample indicating the absence of pentoses.

Characteristics and Properties of the Type-Specific Polysaccharide

The purified S I gives precipitates with uranyl nitrate, basic lead acetate and safranin but not with copper acetate, mercuric chloride,

saturated barium hydroxide, saturated ammonium sulfate, saturated calcium chloride or saturated magnesium sulfate. Samples of S I have been purified by precipitation with basic lead acetate or uranyl nitrate and eluting respectively with sodium carbonate or disodium phosphate. However, the yields were poor and the properties of the product not different from those of the original material.

The S I in the form isolated has no acidic properties. When 100 mg. samples are dissolved in distilled water with a drop of phenol red added, the addition of 0.06 cc. of N/10 sodium hydroxide produces the end-point. The S I is characterized by its high solubility in distilled water and by the difficulty with which it is precipitated. 20 per cent aqueous solutions of S I are readily obtained. In the absence of an excess of electrolyte such as sodium acetate, the S I is not precipitated from aqueous solutions by 10 volumes of absolute alcohol, by 20 volumes of acetone or by 10 volumes of acetic acid. It is not precipitated by 10 volumes of acetic acid even in the presence of sodium acetate. It cannot be precipitated from a solution in 50 per cent acetic acid by adding 8 volumes of ethyl alcohol unless sodium acetate is added also. These facts have made impossible the isolation of an ash-free product.

Attempts have been made to secure an ash-free product by dialyzing solutions of S I in cellophane bags against distilled water. The dialyzed solution was afterwards evaporated to dryness at room temperature and the residue, having been dried to constant weight in a high vacuum in the presence of calcium chloride or sulfuric acid, was analyzed for ash. The recovery of S I was generally poor, possibly due to its adsorption on the surface of the cellophane. The ash content was the same as that of the original material. In one experiment a solution of S I was subjected to electrodialysis using parchment membranes, but since the combined diffusates gave a positive precipitin test with Type I antimeningococcal serum, showing that the S I had diffused through the membranes, the experiment was abandoned.

The S I remains stable at temperatures around 0°C. at least for many months. Thus, preparation 8 was made from broth harvested May 16 and 23, 1933. When the preparation was finished, namely in September, 1933, the titer was 1:8,000,000. On January 8, 1934, a 1:10,000 saline solution was heated for 15 minutes at 100°C. for

sterilization. The product after this treatment still titered 1:8,000,000.

The effect of exposure to various conditions on the precipitin titer of S I is summarized in Table II. In a 1:5,000 solution in *M*/15 phosphate buffer at pH 7.0, the S I withstood a temperature of 100° for 15 minutes. The S I, however, is very sensitive to acid conditions.

Thus, in a 1:1,000 solution in normal hydrochloric acid the precipitin titer had dropped to 1:10,000 after 3 hours at room temperature. In a similar experiment with 0.5 normal HCl, carried out for 1 hour at room temperature, the titer dropped to about one-fifth the value for untreated S I. In a 1:1,000 solution of S I which stood for 23 hours at room temperature in 0.01 normal HCl the effect was not as great but there was a perceptible loss of serological activity. A 1:5,000 solution of S I in 0.2 normal acetic acid-sodium acetate buffer, pH 4.0, was heated for ½ hour at 100°C. and almost all of the serological activity was lost. Accompanying this loss there is an appearance of varying amounts of reducing sugars (7 to 15 per cent calculated as glucose). These appear rapidly (in 15 minutes in the case of the experiment in pH 4.0 buffer heated to 100°C.) and then increase slowly and in 24 to 48 hours may not reach the maximum value of 45 per cent obtained by hydrolysis with strong acid. S I which has not been exposed to strong acid shows no reducing action with the Shaffer-Hartmann reagent.

The exact relationships involved in the acid hydrolysis of S I have not been studied, but preliminary rate measurements have been made, the results of which indicate that a labile prosthetic group is first split off, whereas the bulk of the molecule undergoes a slower degradation. It is hoped to carry out a detailed study of this phenomenon, in order to determine what grouping or configuration is responsible for the serological activity of the S I.

The S I is only slowly broken down by alkali.

The test was a severe one since the dilution of S I was 1:20,000. Heating this in *N*/140 sodium hydroxide at 100°C. for 15 minutes did not affect the serological activity while the use of 0.5 normal sodium hydroxide for 1 hour at 100°C. and of 10 per cent sodium hydroxide for 3 hours at room temperature caused only slight degradation.

The serological activity of the S I is not affected by exposure to nitrous acid at room temperature.

To 1 cc. of a 1:10,000 saline solution of S I were added 0.5 cc. of 30 per cent aqueous sodium nitrite and 0.3 cc. of acetic acid (2.4 times the amount equivalent to the sodium nitrite). A control of 1 cc. of 1:10,000 saline solution of S I with

0.3 cc. of acetic acid was set up. After standing for 3 hours at room temperature with occasional shaking, the solutions were chilled and neutralized with strong sodium hydroxide solution. The results of the precipitin tests showed no dis-

TABLE II
Effect of Exposure to Various Conditions on the Precipitin Titer of S I

Conditions of experiment	Dilution for precipitin test	Precipitin titer with Type I antimeningococcal horse serum
100°C. for 15 min. at pH 7.0	1:50,000	+++ ^{pd}
	1:500,000	+±
	1:5,000,000	±
1.0 N HCl for 3 hrs. at 23°C.	1:10,000	±
0.5 N HCl for 1 hr. at 23°C.	1:100,000	+
	1:1,000,000	±
0.1 N HCl for 23 hrs. at 23°C.	1:100,000	+
	1:1,000,000	±
100°C. for 0.5 hr. in pH 4.0 buffer	1:50,000	±
3 hrs. at 20°C. in NaNO ₂ and CH ₃ COOH	1:50,000	+++ ^{pd}
3 hrs. at 20°C. in 25 per cent CH ₃ COOH	1:50,000	+++ ^{pd}
3 hrs. at 20°C. in 10 per cent NaOH	1:50,000	+++ ^{pd}
1 hr. at 100°C. in 0.5 N NaOH	1:50,000	++
0.25 hr. at 100°C. in N/140 NaOH	1:50,000	+++ ^{pd}
Control, untreated S I	1:50,000	+++ ^{pd}
	1:50,000	+++ ^{pd}
	1:100,000	+++ ^{pd}
	1:1,000,000	++ ^{pd}
	1:5,000,000	+
		±

±
 ±
 +
 +±
 ++
 +++
 } increasing amounts of precipitate.

^{pd} indicates the formation of a disc-like precipitate which can be broken by agitation.

cernible difference between the two solutions and a control of 1:50,000 S I containing the same amount of sodium acetate.

On the basis of the evidence presented, the type-specific substance isolated by the described methods seems to be the sodium salt of a polysaccharide acid composed of a nitrogen-containing sugar and phosphoric acid units. Different lots have uniform composition. The ratio of sodium (calculated on the basis that the ash left upon ignition of S I is $\text{Na}_4\text{P}_2\text{O}_7$) to phosphorus is 1.6 to 1 or nearly 3 Na to 2 P. The ratio of sodium to nitrogen is 3 to 2. The equivalent weights calculated for 1.5 Na, 1 N or 1 P are respectively 325, 321 and 348, corrected to the ash-free basis. The fact that the ash content is not reduced by prolonged dialysis indicates that the sodium is not present as an impurity in the form of salts which might have been carried over in the purification process. The phosphorus is firmly held in organic combination since the molybdate test with S I is negative until the S I has been subjected to vigorous hydrolysis. This conclusion is supported by the fact that the S I gives no precipitate with cupric acetate, barium hydroxide, calcium chloride or magnesium sulfate. The exact form of linkage of the nitrogen has not been determined. It is not present in a condition suitable to give a positive biuret test.

In order to determine whether part or all of the nitrogen contained in S I was present in the form of amino groups, a sample of S I was treated with nitrous acid as follows:

To 2 cc. of a standard solution of preparation 18 (35 to 36 mg. S I) in a 50 cc. centrifuge tube was added 0.3 cc. of a 30 per cent solution of sodium nitrite and 1 cc. of acetic acid. These quantities provided 13 times the amount of sodium nitrite theoretically necessary to liberate the nitrogen present (assuming that all of the latter was in the form of amino groups) and 3 times the amount of acetic acid equivalent to the sodium nitrite. The solution was allowed to stand for a short time and then a slow current of air was drawn through it for 2½ hours at room temperature in order to remove the gaseous decomposition products. The solution was then worked up according to the method described for preparing analytical samples, the product being subjected to two precipitations from its solution in 10 per cent aqueous sodium acetate by the addition of 4 volumes of redistilled ethyl alcohol. The yield was 28.9 mg. (80 per cent recovery).

Analysis showed 9.9 per cent of ash, calculated as sodium, and 4.27 per cent of nitrogen, calculated on the ash-free basis. These

figures are the same, within the experimental error, as those for the starting material (Table I). Consequently, it was concluded that the S I contained no amino nitrogen. Since it has been shown that nitrous acid does not destroy the ability of S I to give precipitates with homologous antiserum, it seems probable that amino groups play no vital part in the grouping responsible for the serological activity of S I.

In view of the findings with respect to the effect of alkali on the specific polysaccharide of the Type I pneumococcus (12), it is realized that the use of barium acetate and sodium carbonate for the removal of sulfate, as described, constitutes a possible weak point in the method of preparation of the S I, although there is some evidence that Na_2CO_3 does not alter the polysaccharide. This procedure may, however, be avoided by the use of prolonged dialysis, and it is thus possible to prepare the S I by mild methods, performing all operations at or below room temperature and never on the alkaline side or more acid than pH 4.0. We have preparations which have not been exposed to alkaline conditions. It is hoped to make a study of these by means of the quantitative precipitin reaction to determine what, if any, changes take place in the S I during the course of purification.

Serological Reactions

All specimens of S I prepared in the method described had a high precipitin titer with monovalent Type I antimeningococcal horse serum. Tables III and IV give typical protocols obtained with the ring test and by the orthodox precipitin test. Specimens react only in very low dilutions with heterologous sera as is shown in Table V. Here both rabbit and horse monovalent sera of Types I and II were used. Salt controls were consistently negative.

That there was complete immunological identity between the different specimens of S I is shown by the following experiment on cross-absorption.

Equal quantities of the various bleedings of Type I antimeningococcal horse serum, which had been absorbed with 0.3 cc. of a 1:1,000 saline solution of S I, preparation 18, per cc. of serum, were pooled. Precipitin tests were set up as usual with this pooled absorbed serum and 1:10,000 saline solutions of preparations 8, 9, 15 B, 17, 18 and 19 of S I. The tubes were allowed to stand for 2 hours at

37°C. and were then mixed and placed in the refrigerator overnight. All were negative. Controls of the S I solutions at 1:10,000 gave a ++++^d reaction against pooled unabsorbed serum diluted with saline to the same strength as the unabsorbed serum.

TABLE III

Serum dilutions.....	1:1,000,000	1:4,000,000	1:8,000,000	Saline
Immediate.....	±R	0	0	0
1 hr. at 37°C.....	+R	±R	±R	0
2 hrs. at 37°C.....	++R	±R	±R	0

Precipitin test with Lot 15 B against third bleeding Type I horse serum using the ring test.

^R indicates the formation of a precipitate at the line of junction of the fluids.

TABLE IV

Serum dilutions.....	1:100,000	1:1,000,000	1:2,000,000	1:4,000,000	Saline
	++	+	±	±	0

Precipitin test carried out with 0.5 cc. portions of antigen and serum in the orthodox method of precipitin tests. The mixture stood for 2 hours at 37°C. and overnight in the refrigerator.

Lot 17 of S I was used and seventh bleeding Type I horse serum.

TABLE V

Serum dilutions....	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	1:2,000,000	1:4,000,000	Saline
Rabbit Type I	++ ^d	++ ^d	++ ^d	++ ^d	+R	+R	0	Negative
Rabbit Type II	±R	0	0	0				
Horse Type I			+++ ^d	+++ ^d	++R	+R	±R	
Horse Type II		0	0					

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RÉSUMÉ

The isolation and preparation of the type-specific polysaccharide (S I) of the Type I meningococcus from hormone broth autolysates is described. As had been noted before, the S I is carried down with the so called nucleoprotein or P fraction at pH 4.0, a reaction which is specific since attempts to repeat it with, for example Type II menin-

figures are the same, within the experimental error, as those for the starting material (Table I). Consequently, it was concluded that the S I contained no amino nitrogen. Since it has been shown that nitrous acid does not destroy the ability of S I to give precipitates with homologous antiserum, it seems probable that amino groups play no vital part in the grouping responsible for the serological activity of S I.

In view of the findings with respect to the effect of alkali on the specific polysaccharide of the Type I pneumococcus (12), it is realized that the use of barium acetate and sodium carbonate for the removal of sulfate, as described, constitutes a possible weak point in the method of preparation of the S I, although there is some evidence that Na_2CO_3 does not alter the polysaccharide. This procedure may, however, be avoided by the use of prolonged dialysis, and it is thus possible to prepare the S I by mild methods, performing all operations at or below room temperature and never on the alkaline side or more acid than pH 4.0. We have preparations which have not been exposed to alkaline conditions. It is hoped to make a study of these by means of the quantitative precipitin reaction to determine what, if any, changes take place in the S I during the course of purification.

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Horse Type I			+++ ^d	+++ ^d	+++ ^d	±R	±R	
Horse Type II		0	0	0	+++ ^d	±R	±R	

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RÉSUMÉ

The isolation and preparation of the type-specific polysaccharide (S I) of the Type I meningococcus from hormone broth autolysates is described. As had been noted before, the S I is carried down with the so called nucleoprotein or P fraction at pH 4.0, a reaction which is specific since attempts to repeat it with, for example Type II menin-

gococcus material, have yielded very indifferent results. This reaction has been utilized. Using untreated broth only about half of the S I was precipitated in this way. More could be obtained by additional ethyl alcohol precipitation but this was unsatisfactory as was, also, the method of concentrating the broth *in vacuo*. However, it was found that preliminary dilution of the broth with an equal volume of tap water, followed by pH 4.0 precipitation, gave good results. The S I was separated from the relatively large amounts of P (20 to 100 times the amount of S I) by repeated fractional precipitation at pH 4.8 or 5.0 in the presence of sodium acetate. Even at this stage the S I shows little cross-reaction with heterologous sera. Additional P was removed with 0.7 saturated ammonium sulfate. The latter can be removed by dialysis through cellophane and precipitation with 10 per cent barium acetate which in turn is removed with sodium carbonate. The S I is obtained from solution by precipitation with 4 volumes of ethyl alcohol and is pure enough for serological purposes. For analysis the last remnant of P is removed with 5 per cent copper acetate and the S I freed of copper by precipitation with alcohol from acid solution. It contains some inactive polysaccharides which may be precipitated out with 4 to 10 volumes of acetic acid.

Analysis of different samples of S I from different Type I strains gave a nearly constant composition with 4.4 per cent nitrogen, 8.9 per cent phosphorus and 9.6 per cent sodium. On acid hydrolysis 45 per cent reducing sugars, calculated as glucose, were liberated. The optical rotation was $+56.8^\circ$ for the sodium D line. Qualitative tests for protein, pentoses, glycogen and starch were negative. The substance was not inactivated by tryptic digestion. The Molisch test was strongly positive in 1:100,000 solutions.

The S I in the form isolated is not acidic. It is highly soluble and precipitable only with some difficulty; is highly sensitive to acid even at room temperature but is unaffected by nitrous acid; and shows but little sensitivity to alkali.

The chemical evidence presented leads to the belief that the type-specific substance is a sodium salt of a polysaccharide acid composed of a nitrogen-containing sugar and phosphoric acid units. The phosphorus is firmly held in an organic combination. The nature of the nitrogen linkage has not been determined. It has been shown that

the S I is unaffected by tryptic digestion and fails to give a biuret test. If the nitrogen is present as amino nitrogen it is not vitally concerned with the serological activity for the latter is unaffected by treatment of the S I with nitrous acid.

Specimens of S I react in dilutions of 1:8,000,000 with Type I monovalent antimeningococcus serum but not higher than 1:100 with heterologous serum. Cross-absorption tests show the immunological identity of the various specimens of S I which have been obtained.

SUMMARY

The Type I meningococcus specific substance has been isolated and purified. It appears to be a sodium salt of a polysaccharide acid.

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THE CELLULAR REACTIONS TO ACETONE-SOLUBLE FAT FROM MYCOBACTERIA AND STREPTOCOCCI

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By KENNETH C. SMITHBURN, M.D., AND FLORENCE R. SABIN, M.D.

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PLATES 38 AND 39

(Received for publication, March 13, 1935)

The lipoidal components of tubercle bacilli have been subdivided by Anderson (1) into phosphatide, so called wax, and acetone-soluble fat. A portion of the wax is unsaponifiable. In contrast to the acetone-soluble fat, the phosphatide and unsaponifiable wax are comparatively homogeneous. Each has been obtained in relatively pure chemical form; the composition of each has also been determined. The cellular reactions induced in animals by Anderson's tuberculo-phosphatide and unsaponifiable wax have been studied by Sabin and Doan (2), Sabin, Doan, and Forkner (3-6), Sabin (7-10), and Smithburn and Sabin (11). As a result of these studies it was found that each of these fractions caused an extensive but relatively simple cellular reaction. The phosphatide induced the formation of tubercle-like masses or nodules of epithelioid cells and Langhans giant cells. These were surrounded by lymphocytes and often showed caseation, although regression of the reaction proceeded by other means. The unsaponifiable wax, when injected into normal animals, caused an extensive reaction characterized by foreign body giant cells, monocytes, young connective tissue cells, and smaller numbers of epithelioid cells. The latter reaction will be reported in greater detail at an early date (12).

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salve-like solid having an odor not unlike that emanating from cultures of tubercle bacilli. On analysis Anderson (1) found this material to be free from nitrogen, phosphorus, and sulfur and to contain large amounts of free fatty acids. Butyric, palmitic, stearic, cerotic, linoleic, linolenic, tuberculostearic, and phthioic acids were present, the latter in large amount. The phthioic acid was identical with that isolated on hydrolysis of the tuberculo-phosphatide. The latter was found by Sabin, Doan, and Forkner (6) to be the only cleavage product of the phosphatide having the capacity to induce the formation of tubercular tissue. It was not surprising, therefore, in the present study to find that epithelioid cells were present in the cellular reaction to the acetone-soluble fat. The cellular responses to this lipoidal component of *Mycobacteria* were discussed in a preliminary report by Smithburn and Sabin (13). In the present communication the details of this study are discussed and illustrated. It will be shown that the acetone-soluble fat is the most irritating of the lipoids studied in this series of investigations. Moreover, it causes hemorrhage and the formation of adhesions, an observation which may throw some light on the occurrence of these conditions in clinical tuberculosis. It will also be shown that much of the irritant action of the acetone-soluble fat is due to its acidity, and may be partly eliminated by neutralization of the lipoid. Neutralization, it will be shown, prevents much of the hyperemia and hemorrhage otherwise induced but does not prevent the formation of tubercle-like structures and adhesions. For purposes of comparison similar studies have been made of the acetone-soluble fat from streptococci, prepared by and received through the courtesy of Dr. M. Heidelberger of Columbia University. The streptococcus acetone-soluble fat likewise induces extensive cellular reaction, which, however, is devoid of epithelioid cells.

Methods

Normal rabbits and guinea pigs were used in the experiments. The lipoid was injected intrapleurally into two animals; in all others intraperitoneally. Some of the animals received a single injection; others daily repeated doses. Since the acetone-soluble fat was insoluble in water, in the first experiments it was suspended in mineral oil for injection. The lipoid suspended in mineral oil was injected into twelve rabbits and two guinea pigs. One rabbit received the melted fat without any vehicle. Later, however, it was found that if the material was

first neutralized with N/10 NaOH, it could then be mixed with water to form a fairly stable suspension. The neutralized acetone-soluble fat or neutralized split products of tuberculo-lipoids were injected into nine rabbits. The effects of the streptococcus acetone-soluble fat were studied in six rabbits. Each animal was killed, at a suitable period after injection, by intravenous injection of air. The fresh tissues were studied by the supravital method; sections were prepared in the usual manner and stained with hematoxylin and eosin.

Mineral oil suspensions of the acetone-soluble lipoids from human, bovine, and avian tubercle bacilli and from *M. phlei* were used in the first studies. Since the lipoids from each of these sources induced indistinguishable reactions, the animals injected with these materials will be discussed as a group. The animals used for each lipoid, the number of injections, interval between last injection and autopsy, and macroscopic characteristics of the reactions induced are recorded in Table I.

Results with the Acetone-Soluble Fats as Such

Since the animals included in Table I showed essentially the same pathologic features, they will be discussed as a group. Each animal (R 2033,¹ R 2036, R 1915—Table I) which received a single intraperitoneal injection of the acetone-soluble fat showed a moderately extensive cellular reaction in the peritoneal cavity. The omentum, mesentery, peritoneum, and diaphragm were especially involved. A single injection caused no adhesions and only moderate hyperemia. The cellular reaction was of very mixed character and in many respects like that occurring after repeated injections, although much less extensive. The predominating cells were in each instance granulocytes, monocytes, and clasmotocytes. In R 2033 and R 2036 (Table I), no epithelioid cells were found in the reaction. This is not surprising as it is known that about 4 days are required for these cells to form. The animals which received repeated injections each showed marked hyperemia of all peritoneal surfaces and especially of the omentum. Fig. 1 shows the marked cellular and vascular reaction in the omentum of Rabbit R 2034, after ten injections. Some animals showed gross hemorrhage. All showed extensive adhesions. Usually the liver, spleen, omentum, and a loop of intestine were bound together into a mass. The omentum or intestine, or both, were in some instances adherent to the parietal peritoneum. Fig. 3 shows marked proliferation of fibroblasts in a section of the parietal peritoneum which was adherent to the omentum and intestines (from Rabbit R 2034). The adhesions were somewhat friable and vascularized. There were yellow colored nodules of cellular reaction and necrosis, 0.1 to 0.6 cm. in diameter, scattered over all the abdominal viscera. The omenta were massively thickened in each instance and very hyperemic. Macroscopic hemorrhage was present in some animals and microscopic hemorrhage in all. The nodules described above were often numerous in the omentum. The retrosternal

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nodes were always more or less enlarged and often hemorrhagic. Peripheral lymph nodes and remote viscera were never involved. Microscopically the cellular reaction was extremely complex. The blood vessels were widely dilated (Fig. 1, R 2034). Often there was evidence of new formation of capillaries. There were always small or large areas of hemorrhage. Every variety of connective tissue cell appeared to be markedly stimulated. There were many granulocytes, undifferentiated connective tissue cells, monocytes, epithelioid cells, clasmato-cytes, lymphocytes, fibroblasts, and plasma cells in the reaction. A characteristic

TABLE I

Animals Which Received Acetone-Soluble Tuberculo-Lipoid

Animal No.	Acetone-soluble fat	Suspended in	No. daily injections	Mg. per dose	Autopsy after last dose	Extent of cellular reaction	Hemorrhage and hyperemia	Adhesions
					days			
R 2173	Human	Mineral oil 0.5 cc.	10	50	1	++++	++	+++
R 2174	"	" "	10	50	2	++++	++	+++
R 2175	"	" "	10	50	5	+++	+	+++
R 2033	Bovine	" "	1	50	1	+	+	0
R 2034	"	" "	10	50	1	++++	+++	+++
R 2035	"	" "	5	50	1 (died)	+++	++	++
R 2036	Avian	" "	1	50	1	+	+	0
R 2037	"	" "	10	50	1	++++	++	++
R 2038	"	" "	2	50	2 (died)	+++	++	++
R 1915	<i>M. phlei</i>	" "	1	50	6	++	+	0
R 2039	" "	" "	10	50	1	++++	++	+++
R 2040	" "	" "	10	50	2	++++	++	+++
R 2794	Human	Undiluted	1	105.6	4	++++	++	+++
R 2099*	"	Mineral oil 0.5 cc.	1†	20	19	++++	+++	+++
R 2100*	"	" "	1†	20	20	++++	0	+++

* Guinea pigs.

† Intrapleural.

area of the omentum of R 2034 showing the complexity of the reaction is shown in Fig. 2. Undifferentiated cells and monocytes predominated, although in many areas there was tremendous proliferation of fibroblasts (Fig. 3, R 2034). Frequently multinucleated cells were present; often there were foci of necrosis. In every animal receiving the repeated intraperitoneal injections the omentum was much too thick for supravital study of films. In these instances scrapings were examined.

One animal, Rabbit R 2794 (Table I), received a single intraperitoneal injection of the acetone-soluble fat undiluted. In this instance the lipid was melted in a syringe, then cooled to about 40°C., and injected, without being suspended in

mineral oil. The reaction was very extensive and differed in no wise from the above. A tubercle-like nodule of cells in the omentum of this animal is shown in Fig. 4.

Two animals (R 2035 and R 2038) died after the injection. In these no intercurrent disease could be detected. Each showed a marked reaction to the material injected.

Two normal guinea pigs (R 2099 and R 2100) each received a single intrapleural injection of the acetone-soluble fat and were studied about 3 weeks later to determine whether or not the adhesions remained. R 2099 showed extensive hemorrhage in the pleural cavity. Both animals showed extensive pleural reaction with adhesions, and consolidation of the lungs on the injected side. The cellular reaction on the diaphragm, pleura, and in the lungs was characterized by marked fibrosis, many young connective tissue cells, and epithelioid cells. The reaction in the lung was obliterative, that is, there was such marked septal thickening that many alveoli were obliterated. Small peribronchial areas of reaction were seen in the lungs on the side opposite the injections. These were composed of the same types of cells but in general the reaction was more purely epithelioid in character. The latter was in all probability due to the fact that the acute reaction had opportunity to subside.

To summarize, it may be stated that the acetone-soluble fat from *Mycobacteria*, introduced parenterally into experimental animals, proved to be markedly irritating and a profound cell stimulant. It may also have some specific toxic properties (death of two animals without other obvious cause). The early reaction was characterized by monocytes, granulocytes, and clasmatoocytes in large numbers with all other connective tissue cells involved to a lesser but not inconsiderable degree. Repeated injections caused hemorrhage and extensive formation of adhesions. When a considerable period of time was allowed to elapse between the last of a series of injections, and the post-mortem examination, the granulocytes and clasmatoocytes played a much less important part in the reaction, whereas the monocytes and epithelioid cells became much more prominent. Most persistent and outstanding features of the reaction, in order of prominence, were: formation of adhesions, hyperemia and hemorrhage, and formation of tubercle-like structures.

In the next studies the acetone-soluble fat was ground in a mortar with sufficient N/10 NaOH to make it neutral to litmus. Saline was added to the desired dilution and further titration to neutrality was done when necessary. The neutralized lipoid was injected into four animals (Table II).

Potassium soaps of the mixed non-tuberculogenic fatty acids from the tuberculo-phosphatide, and of phthioic acid, were prepared for us.² These substances were likewise injected into animals (Table II) and the tissues studied for comparison with the neutralized acetone-soluble fat.

Table II shows the animals which received the neutralized tuberculo-lipoids, the number of injections in each, the interval between the last dose and autopsy, and certain of the macroscopic pathologic features.

TABLE II
Animals Which Received Neutralized Tuberculo-Lipoids

Animal No.	Neutralized lipid	Suspended in	No. daily injections	Mg. per dose	Autopsy after last dose days	Extent of cellular reaction	Hemorrhage and hyperemia	Adhesions
R 2634	Acetone-soluble fat	Distilled H ₂ O	2	50	1	++	+	0
R 2629	" "	" "	10	50	3	++	++	+++
R 2717	" "	" "	10	50	1	+++	+	+++
R 2718	" "	" "	10	50	30	+++	0	+++
R 2412	Fatty acids from tuberculo-phosphatide	NaCl	1	175	15	+	0	0
R 2413	" "	"	10	17.5	3	+	0	0
R 2167	Phthioic acid	"	1	17.5	1	+	0	0
R 2168	" "	"	10	17.5	1	++++	+++	+
R 2169	" "	"	10	17.5	3	++++	+++	+

Results with the Neutralized Acetone-Soluble Fat

R 2634 received two injections and R 2629, R 2717, and R 2718 each received ten injections of the neutralized acetone-soluble tuberculo-lipoid. The cellular reactions in the four were similar. In each instance the reaction was less than in corresponding animals receiving the acid lipid, and of course least in R 2634 which received only two injections. After the neutral lipid the cellular reaction, although reduced in amount and in no instance characterized by gross hemorrhage, was composed of many types of cells. Chief among these were young, undifferentiated connective tissue cells, monocytes, epithelioid cells, and fibroblasts. There were smaller numbers of granulocytes, clasmatoocytes, lymphocytes, plasma cells,

² These soaps were prepared by Dr. R. J. Anderson of the Sterling Chemical Laboratory of Yale University.

occasional giant cells, and small areas of necrosis. Vascular dilatation was present but diminished in amount. Adhesions were just as extensive as after injections of the acid lipid. No epithelioid cells were seen in R 2634, as sufficient time had not elapsed for their formation. R 2629 and R 2717 showed many foci of necrosis about which were intact epithelioid cells. The latter occurred also scattered diffusely throughout the areas of reaction and in small tubercle-like clumps. In R 2718, which was autopsied 30 days after the last injection, the reaction, although still quite complex, was simpler than in the preceding animals. Fewer granulocytes and clasmotocytes were present. Epithelioid cells were present in sizable tubercle-like masses scattered through the omentum, diaphragm, peritoneum, and intestinal serosa. Fig. 5 shows extensive formation of tubercle-like structures composed of epithelioid cells and lymphocytes in the omentum of this animal. In Fig. 6, another area in the same omentum, are two rosette giant cells.

It will be seen that, while the irritant (non-specific) properties of the acetone-soluble fat are in part due to acid substances or groups present in it, the specific tuberculogenic action and the property of inducing adhesions are not eliminated by neutralization. In summary, the three changes brought about by neutralization were: Moderate reduction in the total amount of the reaction, reduction of the vascular irritation, and simplification of the reaction, so that the adhesions and tubercle-like reaction of epithelioid cells were more prominent.

R 2412 and R 2413 which received one and ten injections respectively of potassium soaps of mixed fatty acids from tuberculo-phosphatide, exclusive of phthioic acid, (Table II) showed very little effect from the injections. Macroscopically the reaction was not detected. Microscopically there was a mild reaction characterized by eosinophiles, clasmotocytes, and plasma cells. In R 2413 a single epithelioid giant cell was found in the omentum.

R 2167 received one, and R 2168 and R 2169 each received ten injections of 17.5 mg. of phthioic acid neutralized with KOH. R 2167 exhibited a mild reaction characterized by granulocytes, monocytes, and clasmotocytes with a few small foci of necrosis. The reaction was reflected in the retrosternal lymph nodes. R 2168 and R 2169, which had the repeated injections, each showed far more extensive cellular reaction. The cells were of the same types as in R 2167, but in addition there were many epithelioid cells, both scattered diffusely and arranged in tubercle-like clumps. The reaction in the omentum was most marked in the milk spots, which showed marked proliferation of monocytes and epithelioid cells. The latter were entirely of the type described by Smithburn and Sabin (11) as second and third stage, that is, those with the finer vacuoles. Fig. 7 shows a representative area in the reaction (R 2168) with monocytes, epithelioid cells, and lymphocytes. The reaction in these three animals was more strictly specific than after injection of unneutralized phthioic acid (6).

These results indicate that whereas the irritating properties of the other acids obtained on hydrolysis of tuberculo-phosphatide are almost wholly eliminated by neutralization, the phthioic acid (present in both the phosphatide and acetone-soluble fat) retains all its specific tuberculogenic properties after neutralization. The latter, therefore, confirms the observation that the specific tuberculogenic properties of the acetone-soluble fat remain after neutralization of the latter.

A quantity of acetone-soluble fat³ extracted from streptococci was used in the manner described above, for comparison of the cellular reaction with that induced by the corresponding lipoid from tubercle bacilli. Six animals (Table III) received the streptococcus acetone-soluble fat. At autopsy supravital studies were made, as in the previous experiments, and sections of the tissues were examined. Table III shows the animals which received this material, together with the number and amount of the injections, interval between last dose and autopsy, and certain macroscopic features of the cellular reaction in each animal.

Results with Acetone-Soluble Fat Extracted from Streptococci

Rabbits R 1131, R 2014, and R 2031 (Table III) each received a single injection of 25 mg. of streptococcus acetone-soluble fat intraperitoneally. Each exhibited macroscopically a cellular reaction of moderate extent and of mixed type. Microscopically the reaction was more extensive than had been apparent to the unaided eye. In each instance the predominating cells were polymorphonuclear leucocytes. There were also many clasmotocytes, fibroblasts, monocytes, and undifferentiated connective tissue cells. The omental reaction was diffuse and not limited to the milk spots. There were small foci of necrosis and a few foreign body giant cells. Vascular dilatation was moderate; no hemorrhage occurred, however.

The animals receiving repeated doses showed very extensive and complex reactions. R 1590 received seven injections, R 2015 received five, and R 2032 received three injections. Each exhibited an extensive reaction with hyperemia, necrosis, and adhesions. The extent of reaction was roughly proportional to the number of injections; that is, R 1590 had the most extensive and R 2032 the least extensive reaction. There were fewer granulocytes in the reaction than in rabbits receiving a single dose (R 1131, R 2014, and R 2031, Table III). There was extraordinary proliferation of various connective tissue cells: monocytes, clasmotocytes, fibroblasts, undifferentiated connective tissue cells, lymphocytes, and plasma cells.

³ This was very generously supplied by Dr. M. Heidelberger of Columbia University.

The reactions were quite spotty; that is, in some areas one type of cells predominated, and in other regions another. There were considerable numbers of mitotic figures. The reaction, save for the absence of epithelioid cells and tubercle-like structures, closely resembled that induced by the acetone-soluble tuberculo-lipoid. Fig. 8 shows the marked vascular and cellular reaction in the omentum of R 2015. In only one instance was there any evidence of transport of the material to remote parts of the body. The marrow from the humerus of R 1590 contained a small number of young connective tissue cells arranged in tiny clumps.

It will be seen that the acetone-soluble lipid of streptococci is approximately as irritating as the corresponding lipid from tubercle bacilli. It lacks the power to produce specific tubercular tissue, however, which property of the tuberculo-lipoid is due to its content of

TABLE III
Animals Which Received Streptococcus Acetone-Soluble Fat

Animal No.	Acetone-soluble fat from	Suspended in	No. daily injections	Mg. per dose	Autopsy after last dose	Extent of cellular reaction	Hemorrhage and hyperemia	Adhesions
R 1131	Streptococci	Mineral oil	1	25	2	++	++	0
R 1590	"	0.5 cc.	7	25	3	+++	++	++
R 2014	"	2.0 "	1	25	1	++	++	0
R 2015	"	0.5 "	5	25	1	++++	+++	+
R 2031	"	" "	1	25	2 (died)	+	0	0
R 2032	"	" "	3	25	2	+++	++	0

phthioic acid. In other respects the reactions to the two bacterial derivatives are qualitatively if not quantitatively similar.

DISCUSSION

The cellular responses of the body to invasion by tubercle bacilli with resultant formation of tubercles constitute an important part of the disease process. In the production of the specific lesions the dead bacilli lack only the property of viability to place them on a level of importance with the living organism. Even if the defense mechanism in any given focus or in any individual is sufficient to kill a number of the organisms, unless the latter are promptly removed from the body, a tuberculous lesion is the result. The proteins, polysaccharides, and particularly the lipoids liberated from disintegrating organisms are

toxic or irritating. Necrosis occurs within the lesions; and these necrotic areas appear to be excellent foci for the multiplication of any remaining viable organisms, since it is here that the greater number of organisms are often found. Thus there is set up a vicious cycle: lesions are produced by living and dead organisms, necrosis or caseation occurs, and the result is a focus wherein viable organisms seem to thrive best.

The complexity of the lesions may be in part due to secondary reactions; that is, bacilli which are phagocytized and destroyed may liberate products which in turn destroy cells, further liberating toxic products. Moreover, the chronicity of the disease and of the specific lesions is no doubt due in large part to the resistance of the bacillus itself and of its degradation products, to physical or chemical forces.

Previous investigations have revealed that tubercle bacilli contain a lipid (phosphatide) which is phagocytized by monocytes when injected into experimental animals (11). The monocytes then become epithelioid cells, and tubercles are formed which differ from those in the disease only in that the former contain no bacilli. In the present study it is further shown that the acetone-soluble fat likewise can induce tubercle formation, cause a profound vascular or hemorrhagic reaction, and stimulate the proliferation of fibrous tissue, causing adhesions. The material also possesses marked non-specific irritant properties. That the latter, as well as the hemorrhagic aspect of the reaction, is due to acidity of the lipid is shown by the fact that neutralization reduces or eliminates both phenomena. However, neutralization does not eliminate or diminish the capacity of the acetone-soluble fat to produce tubercular tissue and adhesions. The tuberculogenic property of the acetone-soluble fat is due to its content of phthioic acid; the specific tuberculogenic activity of the latter is not diminished by neutralization.

A study of the cellular reactions induced in normal animals by acetone-soluble lipid from streptococci shows that the latter has no specific tuberculogenic activity; the reaction in other respects, however, is qualitatively and quantitatively similar.

The facts observed in some of the recent studies regarding the behavior of cells toward foreign substances seem about to throw some light on the phenomenon long described as chemotaxis. The behavior

of animal cells toward soluble and insoluble bacterial products, of polysaccharide, protein, and lipoidal nature have been studied. The reaction to a soluble substance is different from that to an insoluble substance; and lipoids, proteins, and polysaccharides each induce reactions easily differentiable from the other. Moreover, two different proteins, or two different lipoids may induce totally different kinds of response. So it is that the chemical make-up of a foreign material (bacterium) may define the nature and characteristics of the response of the animal tissues.

Furthermore, the discovery of chemical units which affect particularly one type of cell may be a means by which the complete life cycle of that cell, together with some of its properties, may be determined (11, 14).

SUMMARY

1. The acetone-soluble fat of tubercle bacilli produces a profound cellular reaction when injected into normal animals. The reaction involves every type of connective tissue cell. Hemorrhage, formation of adhesions and of tubercular tissue occur. The extent of the reaction is roughly proportional to the amount of material injected.
2. The reaction induced by the lipoid is much less extensive and much simpler when the material is neutralized with alkali. Neutralization of the acetone-soluble fat, or of phthioic acid, does not diminish the tuberculogenic property.
3. Acetone-soluble fat from streptococci is likewise extremely irritating but does not produce tubercular tissue.

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EXPLANATION OF PLATES

PLATE 38

FIG. 1. Section of omentum of Rabbit R 2034 after ten daily injections of 50 mg. of acetone-soluble fat from bovine tubercle bacilli. Autopsy 24 hours following last injection. Note very marked cellular reaction and especially vascular dilatation and proliferation. Hematoxylin and eosin stain. $\times 140$.

FIG. 2. Another area in the omentum of the same animal as in Fig. 1. Note complexity of cellular reaction in which there are granulocytes, clasmatocytes, lymphocytes, monocytes, and epithelioid cells. Hematoxylin and eosin. $\times 1300$.

FIG. 3. Marked proliferation of fibroblasts in an adhesion between parietal peritoneum, omentum, and intestine. Rabbit R 2034. Hematoxylin and eosin. $\times 1050$.

FIG. 4. Omentum of Rabbit R 2794 after a single large injection of undiluted acetone-soluble fat from human tubercle bacilli, showing a tubercle-like focus of reaction. The cells in this nodule were epithelioid cells, lymphocytes, and polymorphonuclears. Hematoxylin and eosin. $\times 140$.

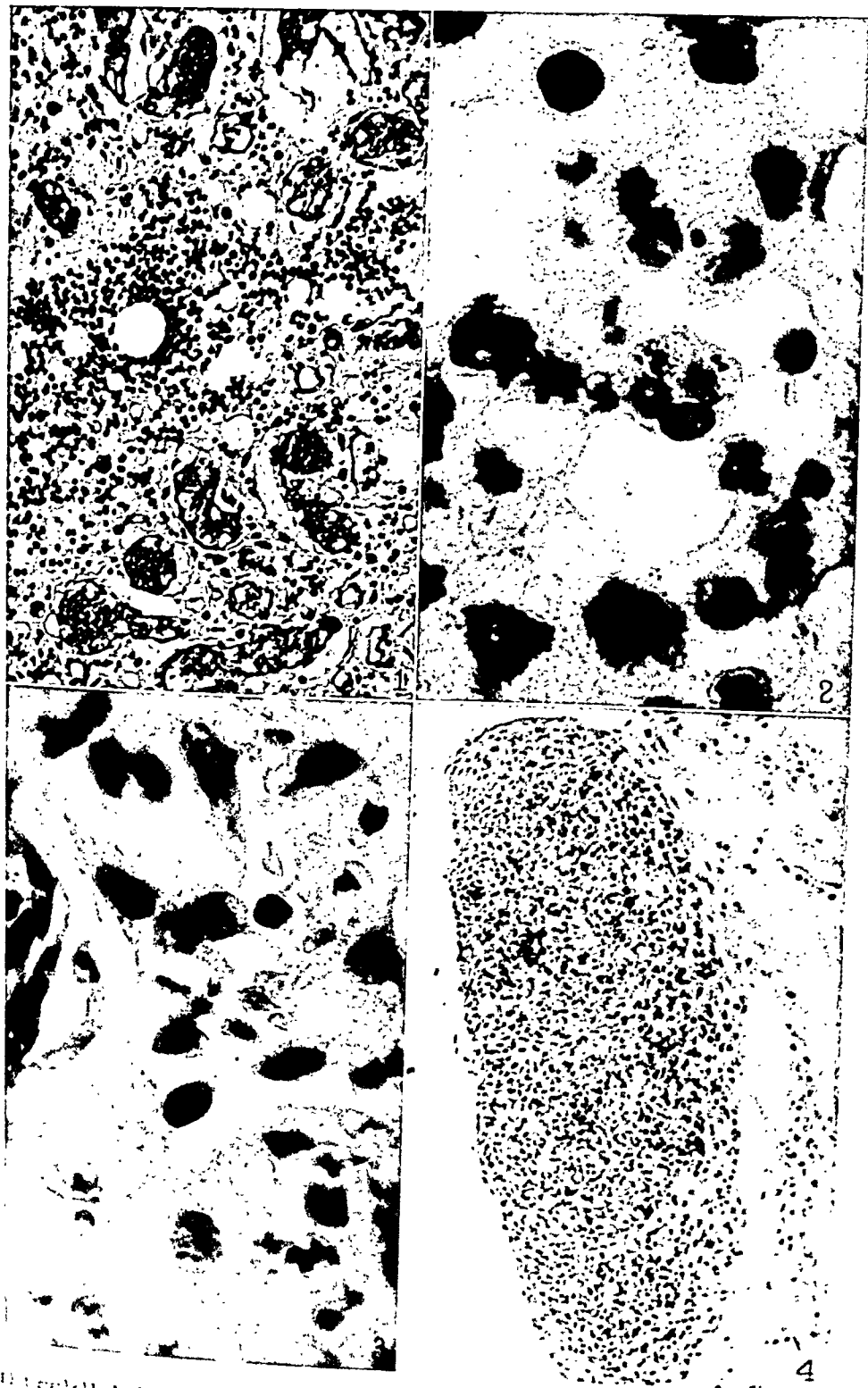
PLATE 39

FIG. 5. Section from the omentum of Rabbit R 2718 after ten injections of 50 mg. each of neutralized acetone-soluble fat from human tubercle bacilli. Note extensive cellular reaction in which there are tubercle-like clusters of epithelioid cells (arrow) surrounded by lymphocytes. Hematoxylin and eosin. $\times 140$.

FIG. 6. Another area in the same section as in Fig. 5, showing two large rosette giant cells. Hematoxylin and eosin. $\times 850$.

FIG. 7. Section from peritoneum of Rabbit R 2168, showing reaction to ten intraperitoneal injections of 17.5 mg. neutralized phthioic acid. Lymphocytes, monocytes, and epithelioid cells predominate in this area. Hematoxylin and eosin. $\times 850$.

FIG. 8. Section of the omentum of Rabbit R 2015 after five intraperitoneal injections of 25 mg. each of streptococcus acetone-soluble fat. Shows marked cellular and vascular reaction similar to that produced by tuberculo-acetone-soluble fat (compare with Fig. 1). The reaction to streptococcus lipid, however, is devoid of epithelioid cells. Hematoxylin and eosin. $\times 140$.



Photomicrographs by Louis Schmidt

Smythorn and Salin: Cellular reactions to acetone, table (6)

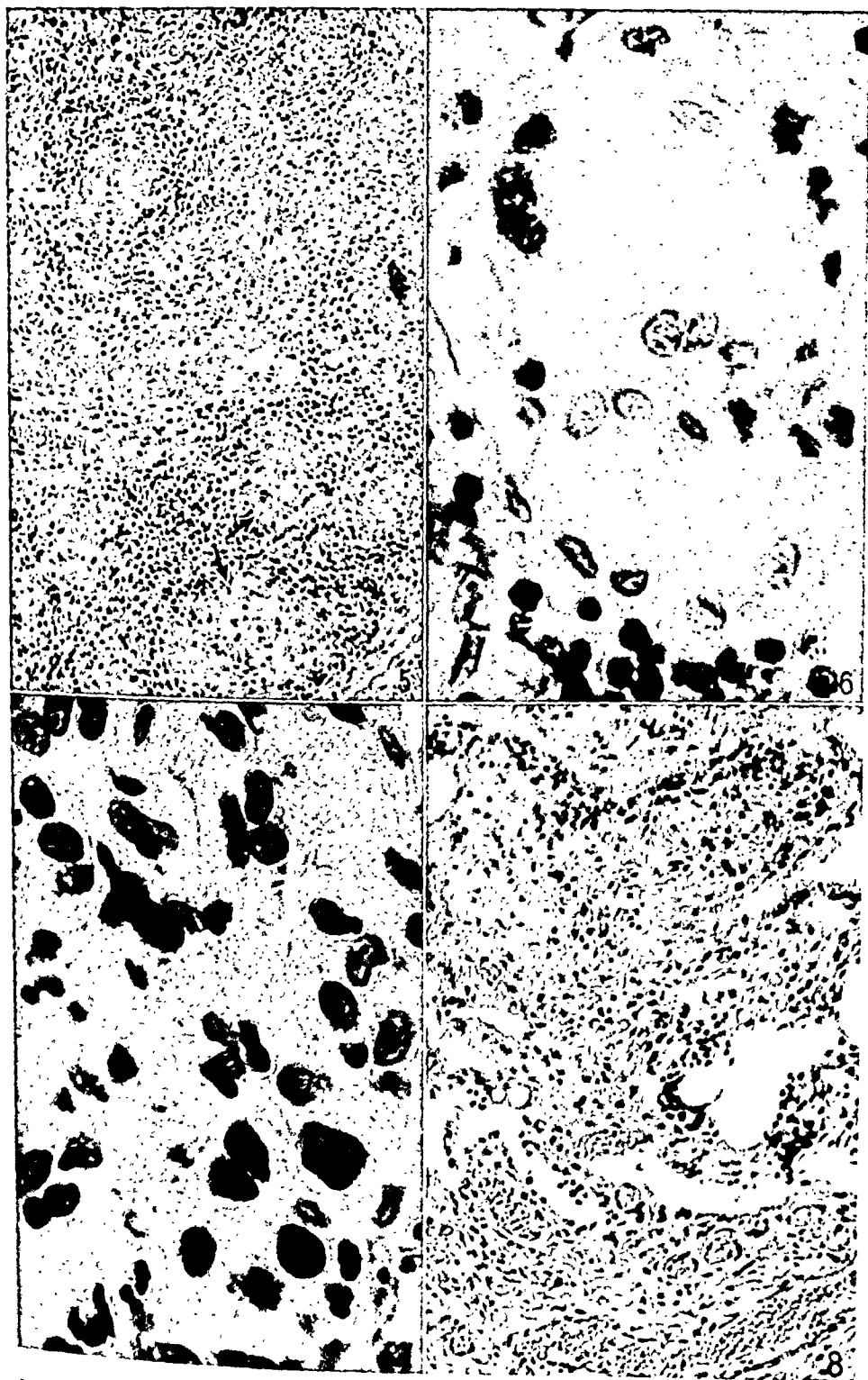


Fig. 39. (5) by Louis Schmitt

THE FORMATION OF AGGLUTININS WITHIN LYMPH NODES

By PHILIP D. McMASTER, M.D., AND STEPHEN S. HUDACK, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, March 19, 1935)

In human skin the superficial lymphatic plexus is so rich that every scratch or puncture wound serves to rupture some of the minute lymphatic capillaries (1). Peripheral lymph flow is far more rapid than is generally supposed (1), and dye substances injected intradermally are carried to neighboring lymph nodes in a few minutes, even in a resting limb (1). Work on the physiology of lymphatics in the ear of the mouse (2) has shown that lymphatic capillaries remain open many hours after an incision. Whenever the continuity of the skin is broken there exists therefore a ready route for infection. Along the path of the infection, between the open lymphatic capillaries of the skin and the entrance of the larger channels into the blood stream, stand the regional lymph nodes; and when such infection occurs with or without lymphangitis they become enlarged and painful. A reaction of lymph nodes to infection is recognized in a multitude of diseases,—as *e.g.* in plague, in typhoid and frequently in tonsillar infection. Pathogenic bacteria carried on the lymph stream are often arrested in the glands through which this stream passes, with result that the infection travels no further.

It seems possible that within the lymph glands there may occur some formation of antibodies to antigens arriving by the lymph stream. The opportunity to test this conception has presented itself to us incidentally to work on the lymphatics of the ear of the mouse. These were rendered visible by dyes and it was found that the extremely rich plexus of superficial lymphatic capillaries in the skin (3-5) drains into one or two common trunks at the base of the ear, which convey the dye to a cervical lymph node, or occasionally a small group of nodes, lying superficially along the margins of the

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thyroid gland. In some preliminary experiments lymphangitis was induced by intradermal injection of typhoid bacterins¹ upon 2 successive days, in the right ears of mice. 4 days to a week later the animals were autopsied and examined. The injections were found to have been largely intralymphatic, and in addition to lymphangitis in the ears there was a great enlargement of the cervical lymph nodes. This way of bringing antigen to the lymph nodes was utilized to find whether these form agglutinins for bacteria.

Methods

Mice of 25-35 gm. body weight were used and approximately 0.02 cc. of the various antigens, to be described below, were injected intradermally into the ears. The injections were repeated at varying intervals as the conditions of each experiment required. To assure ourselves that the injections were truly intradermal, they were carried out beneath the binocular microscope with the mouse under light ether anesthesia and the ears spread on porcelain plaques (3).

At varying intervals following the injections the materials to be tested for antibody content were obtained and extracted in the following manner. The mouse anesthetized with ether or luminal was placed on its back with fore legs slightly elevated. The neck was painted with paraffin oil, and by means of sterile scissors the skin was cut in a T-shaped incision from chin to sternum and then at right angles to this over the axillary vessels on each side. With other sterile instruments the connective tissue was dissected free as the skin was reflected. The procedure disclosed a small superficial lymph node which lies along the cervical vessels near the masseter muscle, receiving lymphatics chiefly from the tongue, and another node or group of nodes lying deeper and more laterally, along the margins of the thyroid gland and receiving lymphatics from the ear. The latter were usually found much enlarged, and were easily shelled out by dissecting away the capsules, scarcely touching the nodes themselves. The mice were then exsanguinated from the carotid artery and the blood specimens pooled for serum.

After weighing the nodes they were aseptically ground with sand, with gradual additions of either sterile 0.2 per cent saline solution or a mixture of equal parts of the latter with glycerine, as the experiments required. In a few experiments only 5 cc. of fluid was used per gram of fresh lymph node material. In most at least 1 or 63 cc. were employed, or more when it was felt the concentration of agglutinins was high. In our first experiments only the glycerine-saline mixture was used, for earlier workers (6, 7) have reported it to be the best extracting fluid for typhoid agglutinins. In the majority of experiments, however, saline solution only was employed and the tests were done immediately. In a few experiments the spleens

of the animals were also ground and extracted. The extracts were centrifuged for an hour at 2500 revolutions a minute in sterile 15 cc. centrifuge tubes, and at times separation of the fatty pellicle was aided by first allowing the tubes to stand in the ice box. The clear central portion of the fluid was removed from the tubes with Wright's pipettes, thus avoiding the fatty pellicle and sediment. The fluid thus obtained was centrifuged again for half an hour and the process was repeated for further clearing. If not yet clear the fluid was diluted further and again centrifuged. It is to be noted that these extracts were made as dilute as possible to rule out non-specific flocculation which might occur during the performance of agglutination reactions with concentrated tissue extracts. No doubt we lost much valuable material in the repeated centrifugations.

Agglutination Reactions.—The cleared lymph node or spleen extracts and the sera of the animals, cleared of blood cells by centrifugation, were employed for titration of the agglutinin content. It was found necessary to do agglutination reactions at once with fresh extracts, for on standing, if only a few hours, a fine fibrinous web developed which could not be centrifuged out. This interfered with agglutination reactions however done and such materials were discarded.

It is well known that non-specific flocculation may be encountered when agglutination reactions are done with tissue extracts. It tends to occur chiefly in highly concentrated extracts, is increased in materials that have been incubated or heated and fails to appear, or does so but rarely, when the agglutination reactions are done at room temperature. We have attempted to avoid it by using tissue extracts diluted as much as possible, by the employment of microscopic agglutination methods in those experiments in which tissue extracts had to be used in final dilution as strong as 1 in 32 or 1 in 64 and, lastly, by the employment in all other experiments of the centrifugation method of Gates (8) which requires no heating or incubation. Thus, in our first experiments (see below) mice were injected only once or twice with small amounts of the bacterins and the lymph nodes on the injected and uninjected sides were removed a few days later. In this early work agglutinin formation was inconsiderable and it became necessary to use tissue extracts more concentrated than 1 in 64 in the final dilutions of the agglutination reactions. We employed the microscopic method for these experiments, confirming the results in three of the experiments by the Gates method, using dilutions as strong as 1 in 64. No non-specific flocculation was encountered. In one other experiment shown in Table VII this method was employed with tissue extracts more concentrated than 1 in 64. In all the other experiments in which dilutions of 1 in 64 or more were employed, as well as in those just mentioned, we have used the centrifugation method of Gates (8). This method requires no incubation or heating and is carried out at room temperature. The titrated mixtures of antigen (heat-killed organisms) and diluted tissue extracts or sera were centrifuged for 10 minutes at a speed just sufficient to deposit unagglutinated organisms, about 1400 to 1500 revolutions a minute. Repeated tests of the temperature of the mixtures before and after centrifugation, whirling the thermometers with them, showed

variations of less than 1 degree and never more than 2.5 degrees. A drop of about 0.5° was usual.

All readings were made in a dark room with the tubes held against a black background in such a way that a beam of light entering at the top gave diffuse illumination of the material at the bottom; and all determinations were made under a binocular microscope magnifying about 15 diameters. After inspecting the tubes and noting the character of the sediment as described by Gates (8), they were all gently shaken in the same way while still under the microscope. The flocculi of agglutinated organisms in tubes showing incomplete or partial agglutination could be seen lying like snowflakes upon the homogeneous layer of sediment. On shaking these rose into the supernatant fluid and appeared like snowflakes, while the more closely packed sediment of unagglutinated bacteria remained at the bottom of the tube and only on further shaking rose in a spiral to be distributed through the fluid in an even cloud. If all the sediment rose as large flocculi and these were found floating in a clear fluid the agglutination was called complete. When large flocculi were found in a slightly cloudy fluid, agglutination was deemed incomplete, and it was considered partial if the flocculi were small. The last tube in which small but strong flocculi indubitably remained when examined 10 minutes after shaking, was considered the end-point.

In the experiments in which microscopic agglutination reactions were done only freshly grown, actively motile cultures were used and the fluids to be titrated were measured with 0.1 cc. quantitative Bureau of Standards pipettes. The fluids mixed in porcelain test tablets were used for hanging drop preparations. All end-points were determined 1 hour after mixing. Saline controls were employed in all tests however done. When agglutination occurred in these the tests were discarded.

The antigens used to induce agglutinin formation were, save where specifically mentioned, heat-killed cultures of organisms suspended in saline. As in all but one instance the organisms belonged to the *Salmonella* group, great care was exercised to avoid all R forms, using only the S forms, because of the superiority of the latter organisms as a source of antigen (9-11). To accomplish this end agar subcultures only 16-18 hours old were used which showed the characteristics of S forms. The killed organisms were suspended in 0.2 per cent saline instead of 0.85 or 0.9 per cent, and, in performing the agglutination reactions the sera and tissue extracts were diluted with 0.2 per cent saline to avoid the non-specific agglutination described by Arkwright (9, 10), and which occasionally occurs with employment of physiological saline solutions.

A further effort was made to standardize the suspensions of organisms employed. The killed organisms in each 10 cc. agar slant were removed in ½ cc. of 0.2 per cent saline, and the suspension, diluted five times further with the same solution, was filtered to break up clumps. A suspension of paratyphoid B bacilli was the first to be made up in this way. Later suspensions of the same or different organisms were brought to approximately the same turbidity by diluting until the appearance of

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fluids in capillary tubes of 2 mm. diameter seemed similar to that of the original.

Orienting Experiments

An emulsion of *B. enteritidis*² was prepared from agar cultures 16 hours old, heated at 65° for 1 hour. The growths were suspended in ½ cc. 0.2 per cent saline for each slant heated and subcultures were made to make sure the organisms were dead. When this had been proven by lack of growth the suspension was further diluted with 0.2 per cent saline to a turbidity similar to that of the paratyphoid bacterin.

TABLE I

Agglutinins for B. enteritidis in Cervical Lymph Nodes and Sera of Mice Injected in Both Ears with Killed Cultures on 2 Successive Days

7 days following the last injection	Dilutions of the gland substance and sera	Lymph nodes	Sera
Group I. Microscopic method	1 in 32	++	+
	64	+	0
	128	0	0
Group II. Centrifugation method	1 in 32	Not done	+
	64	++	0
	128	+	0
	256	0	0
Group III. Centrifugation method	1 in 16	Not done	+
	32	Not done	0
	64	++	0
	128	0	0

++ = incomplete agglutination.
+ = partial agglutination.

A group of 30 mice of about 25 gm. body weight was injected intradermally in both ears with about 0.02 cc. of the *enteritidis* bacterin. 7 days after the last injection the enlarged lymph nodes of the neck were removed from the etherized animals and they were bled for serum. In every instance the nodes were inflamed and much enlarged. The lymph glands from the individuals of this group were ground together and the sera pooled, thus obtaining one node extract and one serum specimen. These were titrated for agglutinins, using the microscopic method as already described. The experiment was later twice repeated and the agglutination reactions done by the centrifugation method of Gates (8) for con-

² Culture obtained from Dr. Leslie T. Webster, The Rockefeller Institute for Medical Research.

TABLE III
Agglutinins in Cervical Lymph Nodes, Sera and Ear Tissue of Mice Receiving on 2 Successive Days Intradermal Injections of B. paratyphosus B. Bacterin in the Right Ear Only

Days after last injection	Dilutions of the tissue substances and of sera	Injected side Right node	Control side Left node	Serum	Injected ear Right	Control ear Left
1	1 in 30	0	0	0	0	0
	60	0	0	0	0	0
	120	0	0	0	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
2	1 in 30	0	0	0	0	0
	60	0	0	0	0	0
	120	0	0	0	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
3	1 in 30	0	0	0	0	0
	60	0	0	0	0	0
	120	0	0	0	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
5	1 in 30	0	0	0	0	0
	60	0	0	0	0	0
	120	0	0	0	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
7	1 in 30	++	0	±	0	0
	60	+	0	±	0	0
	120	+	0	±	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0

+++ = complete agglutination.

++ = incomplete agglutination.

+ = partial agglutination.

± = weakly or dubiously positive agglutination.

TABLE III—*Concluded*

Days after last injection	Dilutions of the tissue substances and of sera	Injected side Right node	Control side Left node	Serum	Injected ear Right	Control ear Left
8	1 in 30 60 120 240 480	++	0	++	0	0
		++	0	+	0	0
		+	0	±	0	0
		0	0	0	0	0
		0	0	0	0	0
10	1 in 30 60 120 240 480	++	0	++	+	0
		++	0	+	±	0
		+	0	±	0	0
		±	0	0	0	0
		0	0	0	0	0
12	1 in 30 60 120 240 480	+++	++	+++	+	0
		+++	+	+++	±	0
		+++	+	+++	±	0
		+++	±	+	0	0
		++	0	±	0	0

ears with accompanying dilatation of blood vessels in the inflamed areas, and it is well known that dilated vessels are more permeable than normal. It follows that agglutinins present in the blood might have escaped into the interstitial tissues of the ear, there to be drained by the lymphatics to the regional node and held in high concentration. Earlier workers, seeking to determine the sites of antibody formation, have injected antigen intravenously and then attempted to demonstrate antibodies in this or that organ extract prior to its appearance in the blood. Their results, like ours thus far recorded, though suggestive of the site of origin of antibodies, fail to prove it for reasons like those just given.

Experiments Demonstrating the Origin of Agglutinins within Lymph Nodes

Three types of experiment were devised to control the possibilities just discussed. In the first type, inflammation was induced in the cervical nodes of both sides and in both ears by the injection of paratyphoid bacterin

on one side and diphtheria toxin on the other. The latter was utilized to produce local inflammation without introducing an agglutinin-forming antigen. In these experiments the ears and nodes on both sides became inflamed and swollen but not quite to the same degree, the nodes on the side injected with paratyphoid bacterin becoming slightly larger. The possible effects of the difference will be considered below.

In the experiment yielding the results recorded in Table IV, about 0.03 cc. of the paratyphoid B bacterin was injected intradermally on 2 successive days into the right ears of 20 mice. Schick test toxin 0.03 cc. was injected intradermally at the same time into the left ears. After 7 days more the animals were bled under ether anesthesia and the nodes on the right and left sides were extracted and titrated for agglutinin content by the microscopic method. Sections of the nodes from both sides appeared similar under the microscope.

As Table IV shows, paratyphoid agglutinins were present in the extract from the right nodes, that is to say, on the side injected with killed paratyphoid organisms. Agglutination was strongly positive at a dilution of 1 to 120 and faintly positive at 1 to 240. Much less agglutinin was present in the serum, tests being positive at 1 to 60 and negative at 1 to 120. No agglutinins were demonstrable in the extract of the nodes on the side injected with diphtheria toxin. The ears on this side were markedly inflamed but slightly less so than on the opposite side.

If, in the earlier experiments, agglutinins formed elsewhere in the body were taken out of the blood by the inflamed nodes on the injected side or had seeped through the permeable blood vessels of the inflamed ear to be drained to the lymph nodes and accumulate there, surely some should have been found in the present experiment on the side on which diphtheria toxin was injected. This was not the case and so the findings indicate that the antibodies present in the right lymph nodes had been formed there.

The experiment still left much to be desired. The nature of the antigens differed, one an emulsion of killed bacilli, the other a cell-free fluid toxin, and it seemed possible that the results might in some way depend upon this difference. For example, in the ear injected with the emulsion of bacilli, many of the organisms no doubt remained *in situ* for a time. Lymph drainage from such an area might con-

ceivably carry antibodies formed in the ear to the regional nodes, there to be concentrated. To control this factor, a second type of experiment was devised.

TABLE IV
Agglutinins for *B. paratyphosus B* in the Sera and in Extracts of the Right and Left Cervical Nodes after Injections into Both Ears

	Dilutions of the gland substance and serum	Right node extract Right ear injected with paratyphoid bacterin	Left node extract Left ear injected with Schick test toxin	Serum
2 intradermal injections on successive days; interval of 7 days			0	+
	1 in 60	++	0	0
	120	+	0	0
	240	±	0	0
	480	0	0	0

++ = incomplete agglutination.

+ = partial agglutination.

± = weakly or dubiously positive agglutination.

TABLE V
Agglutinins for *B. paratyphosus B* in the Serum and in Extracts of Right and Left Cervical Nodes after Injections Into Both Ears

	Dilutions of the gland substance and serum	Right node extract Right ear injected with paratyphoid bacterin	Left node extract Left ear injected with Schick test toxin	Serum
1 intradermal injection only, 2 hrs. later both ears removed; interval of 9 days			0	±
Exp. I. Microscopic method	1 in 60	+	0	0
	120	±	0	0
	240	0	0	0
Exp. II. Centrifugation method	1 in 64	+	0	+
	128	+	0	0
	256	0	0	0

+ = partial agglutination.

± = weakly or dubiously positive agglutination.

Twenty mice were injected intradermally in the right ear only and but once, with 0.03 cc. of the paratyphoid B bacterin. Diphtheria toxin was injected in the left ears. 2 hours later, under ether anesthesia, both ears were amputated. 9 days later the nodes on both sides were removed and serum obtained. In this experiment agglutination reactions were done by the microscopic method. Later it was repeated employing the centrifugation method. Table V gives the results.

of the titrations. The nodes on both sides were much enlarged and inflamed, on the right side slightly more than on the left. Sections of both nodes were similar.

No paratyphoid agglutinin was found in the nodes of the left side, injected with Schick toxin. The extract from the nodes on the other side was positive at a dilution of 1 to 128 in one experiment and weakly so at 1 to 120 in the other. The tests of serum were negative in those dilutions though positive or weakly so at 1 to 64 or 1 to 60 respectively.

This experiment strongly suggests the formation of antibody within lymph nodes, for the nodes on the left side yielded no demonstrable agglutinin; antibody had not passed to them from the blood in spite of the inflammation. The agglutinin present in the nodes on the side injected with paratyphoid bacterin did not derive from the seepage of antibodies from the blood into the interstitial tissue of the ear with subsequent drainage to the node by way of the lymphatics, for the ears had been removed. Complete proof of the formation of antibody by the lymph nodes was not obtained because of the slight difference in the degree of inflammation on the two sides which might be held accountable for the result.

In two other groups of mice both ears were injected on two successive days with the paratyphoid bacterin. 2 days later, at a time when no agglutinins could be demonstrated in either blood or nodes, the left ears were amputated. This was done to prevent the possibility of a seepage of agglutinin in the blood through the walls of the dilated blood vessels of the inflamed ear and its drainage to the node on that side. As a control the right ears were left intact. 6 and 7 days after the last injection, respectively, the two groups of animals were killed and node extracts and sera obtained. Agglutination reactions were done by the microscopic method. As Table VI shows, the titre of agglutinin in the nodes of the two sides was equal, ruling out once again the possibility that the agglutinin in the node was brought to it from the ear. Further, microscopic studies of the nodes from both sides showed no differences.

Proof of the Formation of Agglutinins within Lymph Nodes

A third type of experiment was devised to show finally whether or not agglutinin formation takes place within lymph nodes. Different

antigens of a similar nature were employed; that is to say, suspensions of various killed organisms which would call forth agglutinin formation, and at the same time induce approximately the same degree of inflammation in the injected ears and in the regional lymph nodes.

One bacterial suspension was injected intradermally into the right ears and the other into the left ears. Several days later the titre of both specific agglutinins was determined in the sera and lymph node

TABLE VI

Agglutinins for B. paratyphosus B in the Sera and in Extracts of Right and Left Cervical Nodes and in the Sera of Mice Injected as Described in the Text

	Dilutions of the gland substance and sera	Both ears injected with killed cultures of <i>B. paratyphosus B</i>		Sera
		Right node extract	Left node extract	
2 intradermal injections in both ears on successive days; left ear removed 2 days later; interval of 6 days	1 in 60	++	++	++
	120	±	+	+
	240	±	±	±
	480	0	0	0
Interval of 7 days	1 in 60	++	++	++
	120	++	++	++
	240	+	+	+
	480	±	±	±

++ = incomplete agglutination.

+ = partial agglutination.

± = weakly or dubiously positive agglutination.

extracts from both sides as will be described below. Organisms were used which gave no cross-agglutination.

In a preliminary experiment five series of ten mice each were injected three times, on successive days, intradermally in both ears, with about 0.02 cc. of heat-killed emulsions of five different organisms, *B. typhosus*, *B. paratyphosus B*, *B. enteritidis*, *B. coli* and *B. prodigiosus*, one organism only in each series of mice. The bacterins were prepared in the usual manner from 16-18 hour agar slants of the organisms, killed by heating and diluted until each appeared as turbid as the suspensions of organisms used in the preceding experiments. Every 2nd day, following the injections, two of each series were killed and the lymph nodes removed for comparison. In all a pronounced inflammatory swelling of the lymph

nodes occurred, *B. prodigiosus* producing marked lymphangitis and enlargement. This was to be expected from the recent findings of Rosahn (12), who reported definite pathological changes in mice injected intravenously with *B. prodigiosus*. Two other groups, each of 20 mice, were then injected with *B. enteritidis* and *B. prodigiosus* respectively. After 2 weeks the sera of these mice were tested for cross-agglutination and none found. Sections of the lymph nodes inflamed by the two organisms showed no differences when studied under the microscope. Since similar lymph node swellings were obtained and separately distinguishable specific agglutinins were present in the node extracts, these two organisms were used for the following experiments.

Forty-five mice, approximately 30 gm. in weight, were given three intradermal injections of the two bacterins on successive days. In the right ears 0.02 cc. of *B. enteritidis* suspension was injected and in the left the same amount of killed *B. prodigiosus*. On the 8th day after the last injection the lymph nodes and sera were taken. The nodes of the same side were pooled in two lots, one of which was ground and extracted with 0.2 per cent saline solution, the other with the mixture of equal parts of the saline solution and glycerine. To half the pooled serum glycerine was added in amount corresponding to that present in the node extracts.

Determinations of agglutinin concentrations were made upon the fresh material, using the centrifugation method of Gates (8) (Experiment 1*b*, Table VII), and also the microscopic method (Experiment 1*a*, Table VII). As the end-points of the titrations showed no significant difference between the glycerine-saline and the plain saline solution extracts, only the results with the latter are given in the table.

The highest concentration of *B. enteritidis* agglutinin was found in the extracts of the lymph nodes from the side injected with that antigen, two to four times as much as in the serum. The serum in turn contained more agglutinin for *B. enteritidis* than did the extract of the nodes from the other side. This despite the fact that the nodes were equally inflamed, as judged by their size and appearance in the gross and in microscopic sections. In a corresponding manner the extract of lymph nodes from the side injected with killed *B. prodigiosus* contained the highest concentration of *B. prodigiosus* agglutinin with much less in the serum and least in the lymph node extract from the side injected with *B. enteritidis*.

It is noteworthy that the concentration of each of the agglutinins was greatest in the lymph nodes on the side injected with the corresponding antigen and least in the lymph nodes on the opposite side. The concentration in the serum stood midway between the two. Had agglutinins been formed elsewhere than in the nodes, this distribution could not have occurred.

The experiment was twice repeated with similar results (Experiments 2 and 3, Table VII), using in this and the subsequent experiments the centrifugation method for agglutinin determinations. In another trial a group of mice were four times injected in the same way and examined after a 10 day interval (Experiment 4, Table VII).

TABLE VII
Agglutinins for *B. enteritidis* and *B. prodigiosus* in the Sera and in Extracts of the Right and Left Nodes of Mice Injected in the Right Ears with *B. enteritidis* and in the Left with *B. prodigiosus*

Procedure	Experiment No.	Titre of <i>B. enteritidis</i> agglutinins			Titre of <i>B. prodigiosus</i> agglutinins		
		In extract from nodes on side injected with <i>B. enteritidis</i>	In sera	In extract from nodes on side injected with <i>B. prodigiosus</i>	In extract from nodes on side injected with <i>B. enteritidis</i>	In sera	In extract from nodes on side injected with <i>B. prodigiosus</i>
3 intradermal injections on successive days, 8 day interval after last injection	1a	1 in 256	1 in 128	1 in 64	1 in 128	1 in 512	1 in 1024
	1b	256	64	1 in 32	1 in 64	256	512
	II	256	64	64	64	256	256
	III	256	128	32	64	512	1024
4 injections on 4 successive days, 10 day interval after last injection	IV	1 in 512	1 in 64	1 in 32	1 in 256	1 in 256	1 in 512
	V	1 in 2048	1 in 1024	1 in 512	1 in 1024	1 in 1024	1 in 4096
7 injections in 2 wks., 21 day interval after the first injection							

In a final experiment of the same type, mice were highly immunized by six injections of antigen given within a period of 9 days. 5 days later they were injected once more and a week thereafter (Experiment 5, Table VII), *enteritidis* agglutinins were most concentrated in the extract from the lymph nodes on the side injected with that antigen. The serum contained a high titre of the same agglutinin, almost as high as did the lymph node extract. But the material from

the nodes on the other side showed the least agglutinin for this organism. Almost similar findings resulted from the determination of agglutinins to *B. prodigiosus*. The highest titre occurred in the node extract from the side injected with killed *B. prodigiosus*. It was much less in the serum and in the node extract from the side injected with *B. enteritidis*.

It is to be noted in this connection that in all but one of these experiments (1 *a*, Table VII) the agglutination reactions were done by the centrifugation method, using node extracts in these experiments as strong as 1 in 32 or 1 in 64. As the figures enclosed in broken lines, in columns 5 and 6 show, positive agglutination occurred at this concentration for *B. prodigiosus* in the extract of nodes from the side injected with *B. enteritidis* (column 6), and *vice versa* in column 5. At such concentrations the effect obtained may have been due to non-specific flocculation. However in Experiments IV and V in this table, in which the animals received more injections of antigen and as result were highly immunized, we still found the same effect even at high dilutions. We conclude therefore that our findings are due to true agglutination in all the experiments shown in the table. Should the effect shown in columns 5 and 6 have been due to non-specific flocculation in any of the experiments it would serve as still more definite evidence that agglutinins for a bacterium are formed only in the nodes of the side injected with that organism.

Variations in the Concentration of Agglutinins in Lymph Nodes and Serum

The findings show clearly a formation of agglutinins within lymph nodes. And in the course of the experiments, other evidence was obtained pointing to the phenomenon. The titre of antibody in the lymph nodes and sera varied greatly with the conditions of the experiment. When antigen was injected only once or twice and the concentration of antibody in serum and lymph node extract sought shortly thereafter, far more agglutinin was found in the latter than in the former. But when repeated injections of antigen were made and the interval between the time of first injection and examination was delayed, for example for 12–21 days, the titre of antibody both in lymph node extract and serum was greatly increased but the concen-

tration of agglutinins rose more rapidly in the serum, eventually in one instance exceeding that of the nodes.

Table VIII has been prepared to show this point, using for data experiments which were done with a single antigen, *B. enteritidis*, and in which the agglutination tests were done in the same way, by the Gates method. The findings as just discussed above constitute further evidence for the formation of antibody in the lymph nodes under the conditions of the experiments and its subsequent distribution to

TABLE VIII
Changing Ratio of Agglutinins for B. enteritidis in Nodes and Sera

No. of intradermal injections	Interval from first to last injection	Interval after last injection	Positive agglutination from lymph node substance diluted	Positive agglutination in sera diluted
2 In both ears	Successive days	7 days	1 in 128 64 64 128	1 in 32 32 16 32
	Successive days	8 days	1 in 256 512	1 in 64 64
3-4 In one ear only	14 days	6 days	1 in 1024 512 1024	1 in 1024 512 1024
	11 days	7 days	1 in 1024	1 in 1024
5 In both ears	15 days	6 days	1 in 2048 1024 1024	1 in 1024 1024 2048
5 In both ears				

the serum in ever increasing amounts. No doubt were one to compare the concentration of agglutinin in lymph node extract and serum at still longer intervals, one might find little or no agglutinin in the lymph nodes and much in the blood. From these and from other findings mentioned above, it is plain that one cannot determine the concentration of antibody in blood or organs at one time only and draw conclusions about the site of antibody origin. Early in the process of immunization antibodies may exist in higher concentration

in the lymph nodes than in the blood but late in the process the findings might be reversed.

DISCUSSION

The experiments just described demonstrate the formation of agglutinins by lymph nodes. The finding throws some light upon the phenomena of defense to infection entirely by way of the skin and mucous membranes. In earlier papers from this laboratory (1) we have shown that every scratch, puncture or incision of skin ruptures the minute lymphatic capillaries and that, when ruptured or incised, they remain open (2, 5) for many hours, affording an opportunity for infection. Somewhere along the lymphatic channels body defenses must be active. When vital dyes are placed in superficial cuts (1, 2) they are drained by the lymphatics to the regional nodes. Our present work shows that in the lymph nodes there exists a strong humoral defense mechanism appropriately situated upon the route of the invading infection. Earlier work of others has shown that lymph nodes sieve out large numbers of bacteria injected intravenously or subcutaneously (13-15) and hold them *in situ*. It seems only natural that antibodies should be formed in very high concentration in the organ in which antigen is itself held.

Much has been written of the mechanism by which so called local injections of vaccine or other antigens confer general immunity. Our earlier paper (1) showed that intradermal injections are largely intralymphatic and substances so introduced are rapidly carried by the lymphatics to the regional lymph nodes even in the resting arm. The same must be true when antigen is clinically injected intradermally in a patient's arm to produce a "local" reaction. In view of the findings now reported, one must suppose that some part of the immunity conferred by the procedure is developed through the activities of the lymph glands.

From certain of our findings it seems probable that an intradermal injection need not be wholly intralymphatic in order to incite a formation of antibodies in the lymph nodes. That is to say, lymphatic capillaries need not be broken in large numbers to cause transport of the antigen to the nodes; for apparently lymphatic absorption occurs whether the channels are injected or not. For example, in repeatedly

injecting the ears of our mice, it was noticed that the texture of the skin varied greatly. The thin-skinned ears became badly damaged after a few inoculations and one could no longer effect true intralymphatic injections, yet, to judge by the reaction of the lymph nodes of these animals at autopsy, much of the absorbed material must have been carried to them by the lymphatics, for the nodes were inflamed and enlarged equally with those of tough-skinned animals, in which the injections were definitely intralymphatic.

That local injections produce speedy, remote reactions by vascular absorption is of course a truism as shown by the action of injected drugs; but the rapid lymphatic distribution of antigen is perhaps not so generally recognized. For example, Oshikawa, cited by Hoder (16), injected killed *proteus* bacilli into the ear of a rabbit and 10 minutes afterwards cut off the ear. He sought and found agglutinins in the blood later. Reitler, also cited by Hoder (16), found agglutinins in blood, to *B. coli* and *B. mcsentericus*, when the ear was amputated 3 seconds after a subcutaneous injection. These authors attribute the escape of antigen from the ear to rapid absorption by the blood. From our findings it seems probable that antigen was directly injected into the lymphatics in such experiments and distributed to regional lymph nodes where antibodies were formed. The same result might readily follow intracutaneous immunization as done by Tuft (17). Absorption of some of the residue of an intradermal injection as well as part of the originally injected fluid seems to be by way of the lymphatics.

In the past many authors have sought to determine the place of antibody formation by local or intravenous injection of antigens followed by the extraction of various organs for antibody. Depending upon the site of antigen injection antibodies have been found, now in this organ now in that, in higher concentration than in blood or occasionally before its appearance therein.

As early as 1898, Pfeiffer and Marx (18, 19) titrated the bacteriolysin content of various organs and of the blood of rabbits intravenously injected with killed cholera spirilla. They reported the antibody titre in the spleen, bone marrow, lymph glands, lungs and blood. Bacteriolysin was at times found in higher concentration in the spleen than in the blood and in two or three instances appeared first in the spleen on the 2nd day after injection. Somewhat similar findings were

FORMATION OF AGGLUTININS WITHIN LYMPH NODES

reported by A. Wasserman (20), using typhoid organisms as antigen, and by M. Wasserman employing pneumococci (21).

Following the injection of killed hog cholera bacilli into the mesenteric vein of rabbits, Jones (22) found a higher titre of agglutinin in the liver than in blood or other organs. He showed further that in animals highly immunized the agglutinin titre was highest in serum, but in animals killed early in the process agglutinin was most abundant in the liver. In 1923 (23) Theobald Smith, Orcutt and Little showed that inoculations of *B. abortus* in the various quarters of cows produced agglutinins in the milk and the antibodies were found in highest concentration in the milk coming from the infected quarter. Local antibody formation in the skin has been stressed by Fernbach and Hässler (24), Cannon and Sullivan (25), Cannon and Pacheco (26) and others, and in mucous membranes by Walsh, Sullivan and Cannon (27). Seegal and Seegal (28) showed that the injection of typhoid vaccine into the anterior chamber of the rabbit's eye resulted in a concentration of agglutinins in certain tissues of the eye. Antibodies have been found in varying concentrations in blood, thoracic duct lymph, cervical lymph, cerebrospinal fluid and aqueous humor (29-31). Since the relative concentrations of antibody are similar in actively and passively immunized animals, the findings have yielded no clue as to their place of origin.

Much, too, has been written upon the function of the reticulo-endothelial system in the process of immunization. A discussion of this subject would take us too far afield; but it is conceivable that the cells of this system which are present in the lymph nodes or elsewhere may be responsible for agglutinin formation.

The evidence cited, for local formation of antibodies, though highly suggestive, fails to prove their formation within the tissues investigated: for the mere finding of antibody in high concentration within an organ does not prove its formation therein. The blood vessels in inflamed regions are more permeable than normal and might readily allow antibody to pass from the blood into the tissues with accumulation there. This objection which applies to most of the earlier work of others we have raised against our own experiments in the beginning of this paper, and various devices were employed to overcome it in the later experiments.

That lymph nodes take part in immunity reactions has long been assumed; but almost every tissue in the body and every organ has been considered as the place of antibody formation.

Lymph nodes have long been known to sieve out bacteria (14, 15) and hold them, and definite inflammatory reaction occurs in the nodes when toxins and bacteria are injected intradermally or subcutaneously. As early as 1890 Oertel (32) noticed that lymph glands were affected in diphtheria, that edema of the glands

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occurred and histologically the germinal centers became involved. In 1891 Welch and Flexner (33) inoculated diphtheria bacilli subcutaneously into kittens, rabbits and guinea pigs and described marked changes in the regional glands draining the site of injection. Lesser changes occurred in the lymph glands in other parts of the body. Barbacci (34) confirmed this 5 years later and clinical observations by Bulloch (35) report the finding of enlarged lymph glands in patients with diphtheria.

Councilman (36) called attention to the possible relationship of collections of lymphoid cells to the production of immunity, and, in the following years the significance of these collections of lymphocytes has been much investigated. To judge from the careful studies of Murphy and his collaborators (37) there would appear to be some relation between the lymphocyte and immunity to tumors. Further, Murphy and Sturm (38) showed that rabbits subjected to dry heat showed increased activity of the lymphoid tissue and developed a higher titre of agglutinins to *Pneumococcus* Type I and precipitins to horse serum than did untreated control animals. Other rabbits formed far less of these antibodies following suitable X-ray treatment which reduced the amount of lymphoid tissue.

More recently Takahashi (39) has reported upon the subject of antibody formation in lymph glands. In rabbits immunized against human red cells by intravenous injections over a period of 3 weeks, agglutinins were found in blood serum and in peripheral lymph flowing to and from lymph nodes. This finding is taken as evidence for the formation of agglutinins by the glands themselves. In immune animals, however, agglutinins may be found in practically all the body fluids (29-31) if present in the blood, and the very slight differences found by this worker in the agglutinin content of minute amounts of inflowing and outflowing lymph may easily be accounted for by errors in the method or by changes in the water content of lymph passing through the gland.

For many years, too, morphological evidence has been accumulating to show that the lymphatic system participates in the processes of immunization. Matko (40) described marked changes in the lymph glands within 3 days following vaccination with typhoid "vaccine." Hellman and his coworkers (41, 42) showed that there occurred an increase in the total lymphatic tissues of rabbits of different ages during the process of immunization to paratyphoid bacilli. Antigen was injected intravenously and the spleens showed great increase in size thereafter, especially in their secondary nodules. The lymphatic tissue in the intestine and tonsils increased in amount. From his histological findings Hellman believed that the secondary nodules in the spleen and lymph glands are centers reactive against bacteria and other toxic agents entering the organs. In 1929, Ehrlich (43, 44) described the changes in lymph glands after subcutaneous and intravenous injection of killed staphylococci. Enormous enlargement of the cortex of the lymph glands developed, hand in hand with changes in the number of circulating lymphocytes. Finally the formation of antibodies has been sought in tissue culture by Carrel and Ingebrigtsen (45) who demonstrated the formation of hemolysins by a mixture of bone marrow and lymph glands. Meyer and Loewenthal (46) were unable to produce agglutinins in tissue cultures of spleen, lymph nodes or mesenteric milk

spots when antigen was directly added. However, when living animals were first injected with antigen and the organs removed later and cultured *in vivo* agglutinins were demonstrated.

One further point deserves emphasis. The experiments described in this work were planned to demonstrate whether or not agglutinins are formed within lymph nodes; the findings throw no light on the subject of antibody formation elsewhere in the body. There seems to be no good reason to consider the lymph nodes as the sole site of antibody origin.

SUMMARY

Agglutinins are formed within the draining lymph nodes of mice, following intradermal injections of killed cultures of microorganisms.

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RABBIT POX

III. REPORT OF AN EPIDEMIC WITH ESPECIAL REFERENCE TO EPIDEMIOLOGICAL FACTORS

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Descriptions of an epidemic disease in rabbits comparable with small pox in man were given in a previous paper (1). Pearce, Rosahn and Hu (2) found that the disease was caused by a filterable virus of the pox group which was closely related to vaccine virus but not identical with either the neurotropic or dermotropic form. From their investigations, it may be inferred that the causative agent is either a variant of one or the other or a distinct virus.

The infection produced by this virus is capable of spreading among rabbits and may produce an epidemic much like small pox in man. As was pointed out in previous papers, the epidemic occurred in an animal population which had been under close observation for a period of years and contained a variety of racial types and groups which were known to differ radically in their constitutional potentialities. An unusual opportunity was thus offered for the study of epidemiological factors.

The object of the present paper is to record the essential facts concerning the origin of the epidemic, the source of infection and its spread through the colony and eventual disappearance, together with crude mortality data for different periods of the epidemic. Particular attention will be given to events of epidemiological significance preceding the outbreak. Consideration of selective variations in susceptibility which were disclosed by the epidemic will be reserved for another paper.

Composition of the Population

On Dec. 20, 1932, the colony contained 591 adults and 824 rabbits under 2 months of age. During the first 4 weeks of the epidemic, 291 living young were

born, thus increasing the exposed population to a total of 1706. These animals were distributed among fifteen pure breeds and two groups of hybrids.

With few exceptions, the animals were bred and reared in the colony and no new stock had been brought into the breeding rooms proper since the previous spring. From time to time, however, animals were purchased from outside sources for certain classes of experiments, and these were brought into contact with one division of the breeding colony. Animals under experimental observation and stocks purchased for such purposes are not included in these reports.

Most of the adults were in active breeding service during the fall and until Dec. 16 when all matings were discontinued. From that time until Feb. 17 the male population was idle. During the first 4 weeks of the epidemic, however, there were a number of pregnant females and there were nursing does during the entire period.

The population of the colony was, therefore, mixed, containing racial elements and racial crosses with strong and vigorous groups, on the one hand, and weaklings of various kinds on the other; there was a wide range of age groups, from new born to old age, and the adult members of the population were in various states of physiological activity.

Housing and Care of Colony

The animals were reared indoors and for generations had not been exposed to outdoor conditions of living. They were housed in four steam heated rooms, all filled to capacity. These rooms were equipped with automatic heat control and during the fall, winter and spring, an effort was made to maintain a temperature between 60° and 65°F. The lighting of the rooms was good but ventilation was poor.

Adults were caged separately and, as far as possible, the size of the cage was adjusted to the spacial requirements of individual animals. This was determined not only by the size of the animal, but by temperamental adaptability to spacial limitations.

The cages, in general, were of two classes; namely, individual cages for storage purposes, and breeding and nursery cages. The standard individual cage measured 22 x 14 x 14 inches and, occasionally, a slightly smaller cage was used. Several sizes of breeding and nursery cages were in use; these ranged from a cage 22 inches wide, 30 inches deep and 20 inches high to cages 69 inches wide, 30 inches deep and 26 inches high. In all cases an effort was made to give every animal as much room as possible.

Does are permitted to carry their litters from 4 to 8 weeks, rarely longer. The time of weaning depends in part upon the growth and maturity of the litter and the consequent necessity for nursing, in part, upon the condition of nursing does and the desirability of early weaning as a protection for the doe, and in part on the necessity for using the doe for the production of additional litters. In any case, young animals remain together for a period varying from 6 to 12 weeks. As a

rule, they are separated and caged at an average age of 8 to 10 weeks. In our colony sexual maturity is frequently attained as early as 12 weeks and young animals are sufficiently mature for breeding service at from 4 to 5 months if their development has proceeded normally. This varies, of course, with different breeds and with pure breeds as compared with hybrids.

On account of the composition of the population and the purposes for which the colony is maintained, the use of a diet that is adequate for all animals is neither practical nor desirable. An effort has been made to provide a diet that is adequate for standard, normal groups of animals as determined by criteria of health, reproductive efficiency and the growth and maturity of young stock. This leaves a margin for comparison between control groups and those members of the population whose requirements are greater.

Numerous systems of feeding have been tested but, at the time of the epidemic, we were using a commercial food composed of mixed grains and grain products, chopped alfalfa and mineral salts with a molasses binder. This was supplemented with hay and a free supply of water. No green food was used. This particular diet had been in general use for about a year and on the whole, had given satisfactory results.

The relation of the rooms to one another and to other animal quarters is significant. Three of the rooms occupied the east end of a floor in one building as shown in Text-fig. 1, while the fourth was on the top floor of another building at a considerable distance from the first and connected only by a series of corridors and stairways. This room was in use at the time of the epidemic as an isolation room for animals with chronic or persistent snuffles and acute cases with profuse nasal discharge. When such animals recovered, they were returned to the breeding colony, so that there was a movement back and forth between the breeding colony and the isolation quarters. These animals were, in the main, idle and were not kept under close observation.

Rooms A and B are breeding rooms. They are separated by a wide corridor; both are exposed on two sides. Room A has no communication with any other room, but in B there is a door connecting with C. This was closed by immovable cage racks, but the doorway was not sealed and there was an open space beneath the door.

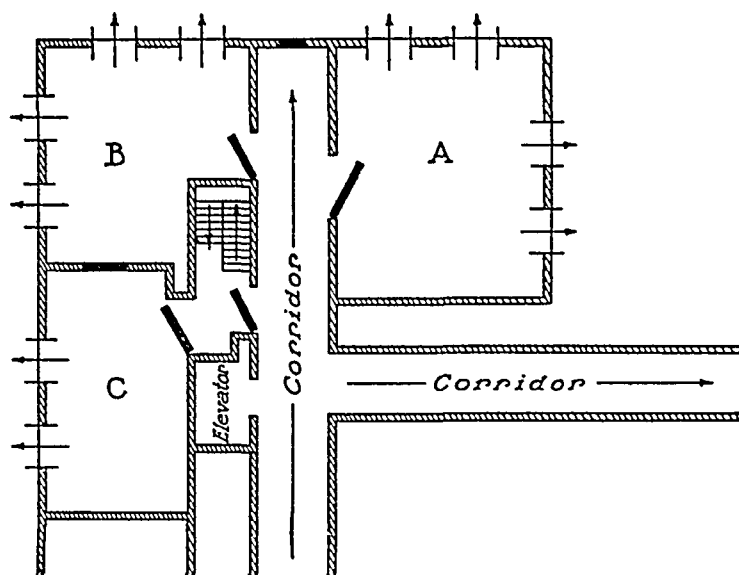
At the time of the epidemic, C was used for miscellaneous stocks, including an overflow from the breeding colony consisting of animals not in active service or of secondary value, and of mild cases of snuffles. In addition, this room contained experimental animals, some of which had been purchased from outside sources. Breeding stocks in this room were examined irregularly or only as there was occasion to use them.

Beyond C, the corridor on which the breeding rooms open continues past the doors of a number of rooms used for housing experimental animals belonging to other laboratories. This corridor is a thoroughfare for the movement of food, bedding, and the litter from cages as they are cleaned.

Rooms A and B were isolated to the extent that they were under the care of men who were assigned no other duties. But the caretaker in C divided his time between that room and others on the same floor, and thus came in contact with other animals. On Sundays and holidays, however, the regular routine was suspended and a reduced crew of caretakers went from room to room to feed and water animals, so that all isolation precautions were broken down on these occasions.

Health Supervision

The health of the colony is subject to close supervision. Animals in the breeding rooms are inspected daily. Cases of illness are noted and mortality records are kept showing the prevalence of disease and the death rate from various causes.



TEXT-FIG. 1. Plan of animal rooms.

These records are supplemented by a weekly enumeration and grading of all cases of snuffles, including animals with moist noses or slightly soiled forepaws as well as those with frank nasal discharges. Functional fitness is gauged by reproductive efficiency based on the percentage of fertile matings, abortions, still births and desertions of litters.

Experience has shown that the prevalence of snuffles and reproductive efficiency are peculiarly sensitive indicators of the health and performance of the colony and that any deterioration or improvement is almost immediately reflected by one or both of these indices, so that the routine management of the colony is based largely on evidence supplied by these records.

*Pre-Epidemic Period**Health and Efficiency of the Population*

In the study of epidemics, information concerning the state of the population during the period preceding the epidemic outbreak is desirable but, as a rule, is difficult to obtain. Observations covering the pre-epidemic period are, therefore, of especial interest and the results of the observations made in the present instance may be presented as events leading up to the epidemic outbreak. These include data on reproductive efficiency, the incidence of snuffles, the prevalence of gastro-intestinal disorders and the death rate from various causes.

Fertility.—As a rule, adult rabbits which have been idle during the summer or for a period of months show evidence of low fertility when first returned to active breeding service. In exceptional cases, the reduction in fertility may amount to complete sterility lasting for a month or more. This condition is accentuated by moult and low fertility is a normal occurrence during moulting periods.

When breeding is resumed in the fall, matings made during the first week or so usually give a small percentage of pregnancies; the normal expectation is from 20 to 40 per cent. The curve then rises to a level of from 60 to 80 per cent and should remain well above 60 per cent. A further rise occurs during the early spring and is followed by a fall coincident with the spring moult and the beginning of hot weather. The exact figures are influenced by the frequency with which persistently sterile animals are used and, in compiling fertility records, corrections are made on this account and no matings are used unless service is actually observed.

The records for the fall of 1932 are given in Table I and Text-fig. 2. Breeding began the last week in September and continued without interruption until Dec. 16. It will be seen that the fertility curve followed a normal course and reached a high point of 71 per cent for matings made during the first week of November. This was followed by a decided drop which, in the routine examination for pregnancy, 10 days after mating, was not detected until the middle of November. At this time, other signs of deterioration were apparent.

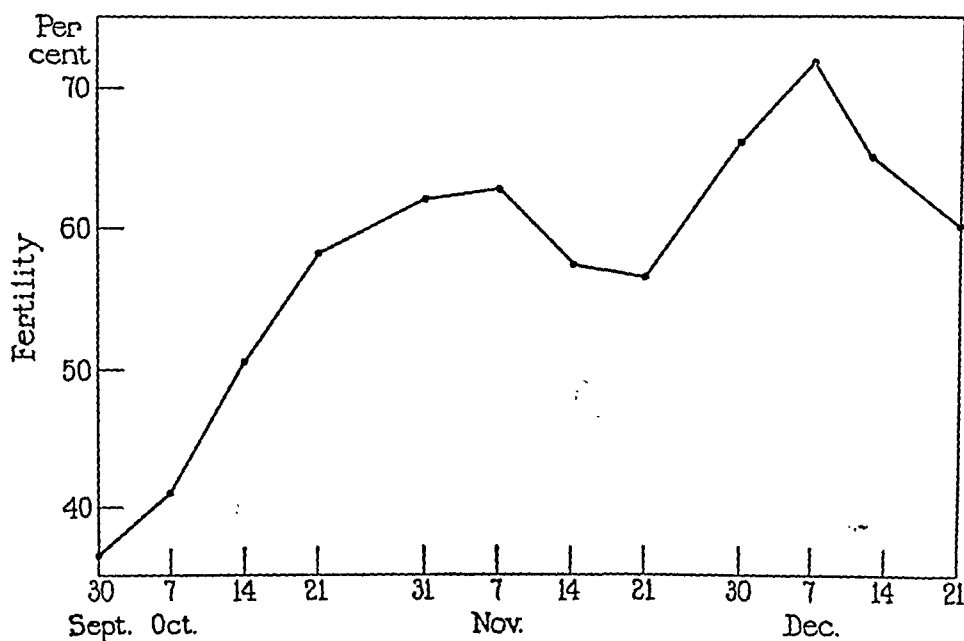
Similar disturbances had been encountered on other occasions and, while their true cause was not known, it had been found that they could be corrected by the administration of vitamin concentrates in excess of the normal requirement. The adult members of the population were, therefore, given brewers' yeast, cod liver oil with viosterol, and tomato juice by hand for a period of about 2 weeks. This was a laborious task and could not be carried out indefinitely and, as improvement was not as rapid or as satisfactory as usual, arrangements were undertaken for the incorporation of additional vitamins in the regular food ration. This procedure

could not be put into operation before the epidemic of pox occurred. Meantime, selective vitaminization of the most seriously affected and most valuable animals was continued.

Reference to the fertility curve in Text-fig. 2 shows that there was a definite response to vitaminization. The curve rose from a low level of 51 to 56 per cent

TABLE I
Fertility during the Pre-Epidemic Period

	Sept.	Oct. 1-7	Oct. 8-14	Oct. 15-21	Oct. 22-31	Nov. 1-7	Nov. 8-14	Nov. 15-21	Nov. 22-30	Dec. 1-7	Dec. 8-14	Dec. 15-21
Matings.....	46	96	102	81	72	49	39	43	35	39	50	10
Pregnancies.....	16	38	51	51	41	35	20	24	22	32	30	6
Fertility, per cent.....	34.8	39.6	50	63	57	71.4	51	56	63	82	60	60



TEXT-FIG. 2. Fertility during the pre-epidemic period.

during the 2nd and 3rd weeks of November to 82 per cent for the first week of December. Routine vitaminization was discontinued at this time and the curve again dropped abruptly.

Abortions.—The gestation period in the rabbit varies from 30 to 32 days with a mean of approximately 31 days. Pregnancy can be diagnosed in some instances by palpation of the abdomen as early as the 8th day following coitus and, with rare exceptions, can be diagnosed with certainty by the 10th day. The term abortion

is used here to cover the loss of litters during the 21 day period after pregnancy has been established. Nothing definite is known concerning earlier interruptions of pregnancy. Abortion usually occurs during the last week of pregnancy with the birth of a premature litter, either living or dead. Occasionally, it occurs earlier and, in such cases, the products of conception are usually eaten by the doe so that the exact date of abortion cannot be determined.

No abortions occurred among the animals mated during September or the first 3 weeks of October, but the rate for subsequent matings rose from 5 per cent during the last week of November to 7.3 per cent during the 2nd week of December. From this point on the picture was complicated by the prevalence of pox. The abortion rate rose abruptly and progressively after the outbreak occurred, reaching the high point of 83.3 per cent among does mated just before the epidemic began. It is important to note that the first increase in abortions occurred among animals of the group in which a decrease in fertility was first recognized. Thus, decreasing fertility and abortion are linked together as immediate and ultimate responses to some disturbance in the animal organism.

Still Births.—In checking litters at birth, dead macerated fetuses and animals that are immature and evidently dead before birth are occasionally found. But, as a rule, it is impossible to distinguish between deaths which occur just before, during or immediately after birth. All of these represent birth losses and are classed as still births. The number of such animals varies normally in different breeds or families; in some they are of common occurrence and are most frequent among animals known to transmit certain constitutional abnormalities. Still births usually average between 2 to 5 per cent of the total number of young born and are limited to few litters.

No significant deviation from the expected percentage of still births was found during the pre-epidemic period, but toward the end of this period, there was a definite increase in the proportion of litters containing still born young. That is to say, the condition was more widespread than usual.

Desertions.—Neglect, desertion or the destruction of litters at birth or during the first week of life is a phenomenon of considerable interest. The preparation for the birth of a litter and the care given the litter at birth and during the first few days thereafter are expressions of racial, familial and individual characteristics, and any deviation from the usual course of events in a given animal is a peculiarly sensitive index of the true state of health of that animal. To appreciate this fully, the individuality of the animal must be known, but the failure of any animal to perform these functions adequately, or the manifestation of cannibalism, may be accepted as conclusive evidence of abnormality. In some instances, it is a disinclination, in others an inability to care for the young and in still others a morbid appetite which leads to desertion on the one hand, and cannibalism on the other. In the great majority of cases these expressions of abnormality can be abolished by appropriate treatment (3).

An analysis of maternal behavior in relation to losses by desertion is too compli-

cated to be attempted here. It is sufficient to say that during October and November desertions were only occasionally encountered, and most of these occurred in a family of closely inbred animals which rarely nurse their own young but, as a rule, will rear foster litters. Throughout the remainder of the pre-epidemic period, however, there was a progressive increase in desertions on the part of does that normally raise large, healthy litters, and this included some of the most dependable animals in the colony.

This was regarded as convincing evidence of the prevalence of some condition which was undermining the health of the colony and was the main reason for the decision to stop breeding operations on Dec. 16, or about 2 weeks before the first case of pox was found in the breeding colony. It will be noted again that this manifestation of abnormality occurred in the same group of animals previously identified with diminished fertility and increasing abortions. While these three measures of the functional status of the female population of the colony are separated in time of expression, they are all connected with some disturbance in the health of the community dating back to the middle of November, or more than a month before the outbreak of pox occurred.

Prevalence of Snuffles.—In considering the significance of snuffles as an index of health, it should be pointed out that the great majority of rabbits are carriers of *Bacterium leprosepticum* or *Bacterium bronchisepticum*, or both. So called snuffles-free colonies may be established and maintained by employing suitable precautions. However, no such precautions were taken with this colony as we preferred to utilize snuffles as a constitutional index. Considerable time has been devoted to study of the disease with especial reference to the factors which influence susceptibility.

Briefly, it has been found that, in the main, snuffles is a disease of adolescent and adult life. Nursing young are peculiarly insusceptible, but susceptibility increases with weaning and reaches a maximum at puberty. After full sexual development is attained, the presence or absence of snuffles is a function of health and vigor.

The incidence of the disease varies greatly in different breeds or family groups. There are some groups of animals in the colony which are so susceptible that, with rare exceptions, every animal develops a nasal discharge by the end of the 5th month of life. On the other hand, there are some in which snuffles is rarely seen at any time. Between these extremes, however, snuffles is variable and is subject to the influence of numerous factors. For example, moulting is always preceded or accompanied by an increase of wet noses or frank nasal discharges. Males that are used too often for service and does that are bred too frequently or made to carry litters too long are apt to develop snuffles. On the other hand, idle females that are excessively fat and indolent are frequently affected. In such cases the condition is often relieved by pregnancy and lactation.

A similar situation obtains with respect to spacial requirements. With many animals, snuffles is merely a response to overcrowding or to too close confinement

in small cages and can be relieved by a suitable readjustment of cage accommodations. Overheating of rooms and unseasonable weather are also factors of importance.

The presence in the colony of animals with frank nasal discharges is a factor of uncertain significance. A limited isolation has been practiced as a presumptive measure of protection to young stock, but it has never been possible to remove all animals with nasal discharges from the breeding rooms. There is, however, no evidence to show that the presence of such animals has any material influence on the actual prevalence of snuffles.

The evidence available indicates that the prevalence of snuffles represents an expression of inherent susceptibility on the one hand, and the action of environmental factors which affect the functional efficiency and the health of the animal on the other. In our colony, it is a disease of no particular consequence except in certain racial groups, and rarely are other cases encountered which will not respond to simple methods of dietary and hygienic treatment.

TABLE II
Incidence of Snuffles during Pre-Epidemic Period

	Oct. 22	Nov. 9	Nov. 15	Nov. 21	Nov. 28	Dec. 7	Dec. 14	Dec. 21
Snuffles, <i>per cent.</i>	14.7	13.9	12.0	13.6	13.6	15.5	17.4	23.6

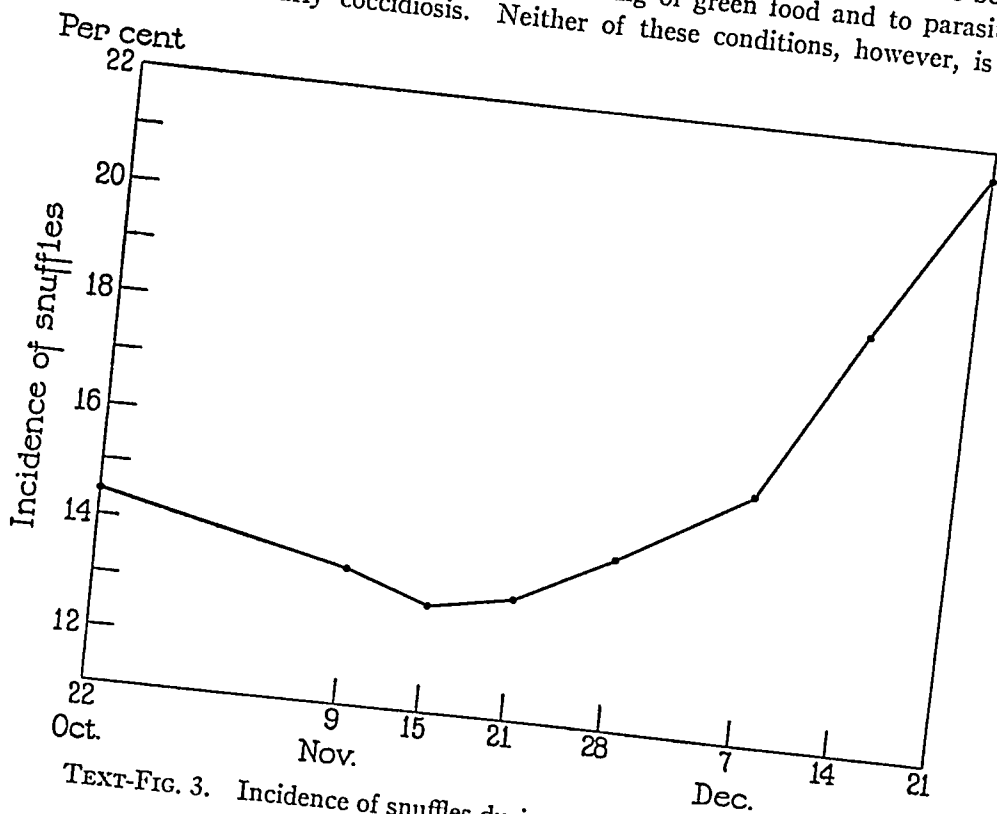
The incidence of snuffles from Oct. 22 to Dec. 21 is given in Table II. These figures are based on the adult population and include every case of wet nose in the colony. The values for the first three checks represent a basic level and are attributable mainly to animals with more or less persistent snuffles. The slight decline shown on Nov. 15 was referable, in large part, to the weeding out of some of these animals; the few new cases that developed were offset by recoveries. Other animals were discarded later, but there was a gradual increase in new cases, culminating in a sharp rise during the 2nd and 3rd weeks of December when nearly a fourth of the colony was affected. During this period the severity of the disease increased, cases began to appear in the young stock and there was a rise in the death rate from pneumonia in both classes of animals. After Dec. 21, snuffles checks were discontinued as cases of simple snuffles could not be distinguished with certainty from incipient cases of pox.

This series of observations again indicated the presence of some disturbing influence in the colony which, in this instance, assumed the form of a definite increase in the incidence and severity of an upper respiratory infection and an increase in the death rate from pneumonia. This sharp rise occurred more than 2 weeks in advance of the outbreak of rabbit pox and coincided with an equally sharp decline in the fertility of the colony as shown in Text-figs. 2 and 3.

Gastro-Intestinal Disorders.—Gastro-intestinal disorders varying from mild and transient cases of slobbers and diarrhea to chronic progressive and acutely fatal

affections constitute the most serious group of diseases with which we have had to contend. Affections of this class are most frequent in young animals and account for the great majority of deaths during the first 3 or 4 months of life. They are less serious in older animals but, at times, the mortality in adults is also high. No age group is exempt and even animals 1 to 3 weeks old, dependent entirely upon nursing, are subject to periodic outbreaks of a highly fatal nature.

The etiology of this class of disorders is uncertain. By some they have been attributed to improper feeding or to the feeding of green food and to parasitic infections, particularly coccidiosis. Neither of these conditions, however, is a



TEXT-FIG. 3. Incidence of snuffles during the pre-epidemic period.

factor of primary importance in our colony; coccidiosis is extremely rare and green food is not used. From investigations extending over a period of years, it has been found that, on a standard diet, gastro-intestinal disorders are of variable frequency and severity. Incidence and mortality are highest in hot weather, but for unknown reasons serious outbreaks occur at other times. It has been found also that, in certain stocks, gastro-intestinal disorders are rare at any time while, in others, incidence and mortality are comparatively high at all times and under all circumstances. Diet in the form of vitamin concentrates or the use of a suitable foster mother is the most potent factor in influencing these affections experimentally. In brief, the information obtained so far indicates that there are at

least two groups of factors concerned in the periodic outbreaks of these disorders, the one an hereditary predisposition and the other a variation in dietary requirement which is determined by unknown environmental factors.

Mortality records for the period preceding the outbreak of rabbit pox show that there were no deaths from gastro-intestinal disorders prior to Nov. 16. There was one death in a young animal on this date from acute bloat, another on the last day of the month and two the first week of December (Table III). This is a low normal expectation and covers a period in which there was not a large number of highly susceptible young animals in the colony. During the 2nd and 3rd weeks of December, the rate rose rapidly and there were six deaths among adults. This rise continued for 2 weeks after the first case of pox appeared and then decreased as the epidemic reached its height. These relations are shown in Text-fig. 4.

TABLE III
Mortality from Gastro-Intestinal Disorders in Young and Adult Stock

	Nov. 16-22	Nov. 23-29	Nov. 30- Dec. 6	Dec. 7-13	Dec. 14-20	Dec. 21-27	Dec. 28- Jan. 3	Jan. 4-10	Jan. 11-17	Jan. 18-24
Young stock										
Deaths.....	1	0	3	4	25	35	43	26	8	2
Mortality, per cent.....	0.13	0	0.37	0.49	3.07	4.32	5.62	3.88	1.69	0.69
Adult stock										
Deaths.....	0	0	0	0	6	4	0	0	1	0
Mortality, per cent.....	0	0	0	0	1.01	0.68	0	0	0.19	0

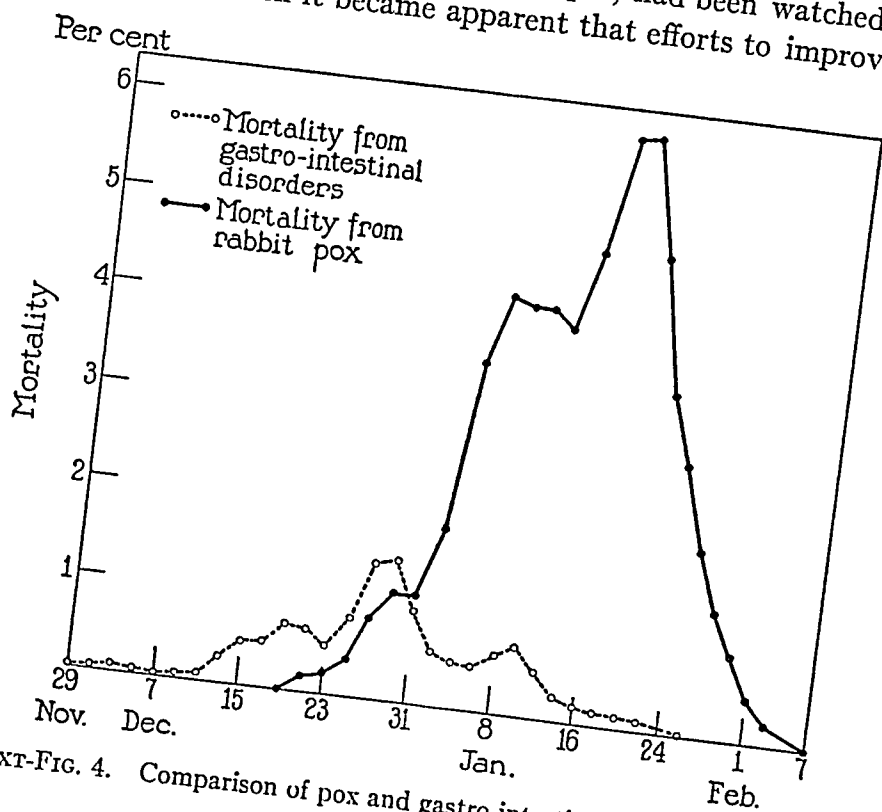
Miscellaneous Signs of Deterioration.—Besides the criteria of health mentioned above, there were other indications of a widespread and profound disturbance of the health of the colony which cannot be recorded in detail. It may be said, however, that there was a recurrent moult among the adults in late November and early December. Matings were obtained with difficulty due to a depression of sexual characters of both males and females. At the same time young animals showed retarded growth and absence of customary thriftiness. Signs of retarded and abnormal development, such as primary nakedness or subnormal development of the hair with deficient pigmentation which are usually limited to certain family groups, were found widespread in full term litters.

Birth abnormalities such as calcification defects in the bones of the calvarium or complete absence of calcification with resulting hydrocephalus and abnormal development of the jaws with malocclusion of the incisor teeth are of sporadic occurrence in certain groups of constitutionally defective animals. These conditions have a genetic background, but their expression is largely determined by environmental factors. In other animals, functional disorders of a lethal character

occur with more or less regularity, but the time of appearance and the severity of these affections are materially influenced by environmental factors. During the pre-epidemic period, birth abnormalities occurred with increased frequency, while lethal deficiencies progressed more rapidly to a fatal termination.

The Epidemic

The unfolding of these signs of physical and functional deterioration, at first gradual and then more rapid, had been watched with great concern. When it became apparent that efforts to improve the



TEXT-FIG. 4. Comparison of pox and gastro intestinal mortality rates.

condition of the population by dietary and hygienic measures were not attended with the usual success, breeding operations were suspended as a last resort. This was done on Dec. 16 and the first typical case of pox was found in a young rabbit in Room B on Dec. 28.

The infection spread rapidly through this room with cases developing simultaneously in all locations. There was no evidence of spread by contact or proximity from a primary focus of infection but, at the outset, the main focus appeared to be in the rear of the room adjoining

ing Room C, and during the early part of the epidemic, the incidence of infection and mortality was highest in this group of cages.

Scattered cases of pox were found in A soon after they appeared in B. In this room, however, which contained a larger proportion of adult animals and was less crowded than B, the infection spread more slowly and the peak of the outbreak was not reached until the disease in B was well on the decline. There were comparatively few deaths in C and, for the most part, those in D occurred before the nature of the disease had been recognized.

The incidence of new cases could not be followed and recorded day by day. The only accurate record of the course of the epidemic is derived from the mortality records. Dead animals were collected daily, records of their death were made and autopsies performed. The data obtained in this way are given in Table IV and Text-fig. 4.

TABLE IV
Pox Mortality

	Dec. 20-25	Dec. 26-31	Jan. 1-6	Jan. 7-12	Jan. 13-18	Jan. 19-24	Jan. 25-30	Jan. 31- Feb. 5
Deaths.....	7	45	127	139	166	93	28	5
Mortality, <i>per cent.</i>	0.5	3.3	9.8	12.4	17.4	12.2	4.0	0.7

The epidemic, recognized as such, began Dec. 28 and ended Feb. 5. It will be noted, however, that the record begins with Dec. 20, more than a week before the first clinically typical case of pox was found in the breeding rooms. A review of autopsy protocols in the light of subsequent experience showed that deaths from pox, clinically unrecognized because of the absence of external lesions, occurred in Room B on that date. Deaths which occurred in Room D as early as Dec. 12, and at the time were attributed to pneumonia, probably resulted from pox infection but are not included. Moreover, there were deaths during February and even later which were due indirectly to rabbit pox, but none of these are included in the data given above. The mortality curve for the period (Text-fig. 4) is typically that of an epidemic. The only irregularity is shown at Jan. 12 and this is referable to the superimposing of what amounts to two closely related epidemics, the one in Room B and the other in Room A.

Of the 1706 animals constituting the population during the epidemic period, 593 were killed or died of various causes including desertion, diarrhea and miscellaneous disorders and are not included in the determination of the pox mortality. During the period from Dec. 20 to Feb. 5, 610 animals died of rabbit pox, giving a mortality of 46.4 per cent. Many others were so seriously affected that they subsequently

died or were killed in the reorganization of the colony. Actual losses attributable to the epidemic were, therefore, much higher than the figures given above would indicate.

In discussing the origin of the epidemic, reference will be made to peculiar affections which preceded and followed the epidemic outbreak. It is not known whether these conditions were referable to pox infection, but it is certain that the epidemic began and ended with cases of infection which could not have been recognized as pox except by their association with typical cases of the disease. Thus, the first deaths were ascribed to pneumonia of a virulent type. These animals showed a profuse blood-stained discharge from the nose with massive consolidation of the lungs, but no characteristic focal lesions as in typical cases of pox. The evidence available, however, indicates that these were cases of pox infection. Moreover, during the 4th week of December, there occurred a number of deaths in Room B among young animals which showed signs of an acute gastro-intestinal disturbance, but no clinical evidence of pox infection. At autopsy, however, the focal lesions afterwards found to be characteristic of the disease were discovered in their internal organs. The first death of this kind occurred on Dec. 20 and it was more than a week before typical external lesions appeared in affected animals. Toward the end of the epidemic, on the other hand, mild cases of infection were common with a tendency to monosymptomatic affections difficult to recognize or easily confused with some other disease. Prominent among these were cases of keratitis and iritis with or without the production of glaucoma.

It can be said, therefore, that the time of the actual appearance of the infection in the colony and of its final disappearance are unknown. The disease began insidiously and died out in an equally obscure manner, leaving behind a train of affections suggestive of pox but not definitely established as cases of pox infection. On the basis of the distribution of lesions, three phases in the history of the infection could be recognized. At first the disease was entirely visceral, appearing as a fulminating pneumonia on the one hand and as an acute affection of the gastro-intestinal system with numerous focal lesions on the other. Later, with involvement of the skin and all organ systems, the infection assumed its typical form. Toward the termi-

nation of the epidemic, visceral lesions were less numerous and manifestations of infection were limited almost entirely to the skin.

Origin and Spread of the Infection

When the condition of the colony became so serious that we were forced to discontinue breeding operations, a systematic examination of all animals was made for evidence of any obscure disease which might have been overlooked in the course of routine observation. These examinations were continued day by day with attention centered on the breeding rooms, but for some time nothing definite was found. Suspicion was first aroused by a sudden increase in the mortality from what appeared to be pneumonia among the animals with snuffles in the isolation room (D). These animals became acutely ill; some showed a profuse blood-stained discharge from the nose or the formation of thick brown crusts about the nares with marked respiratory distress followed by death in a few hours or by gradual recovery. At autopsy the outstanding condition was a massive pneumonia. There were no typical pox lesions, either external or internal. Examination of several males in process of recovery showed, however, that these animals had a granular orchitis undergoing resolution.

The significance of this finding was not appreciated at the time, but it was known that we were not dealing with an ordinary pneumonia. These were undoubtedly atypical cases of pox. As was mentioned above, the animals in this room were not subject to systematic observation and the time of occurrence of pox in this group can be fixed only with reference to the flare up of pneumonia. The first death of this kind occurred Dec. 12. Typical cases of pox were never found among the breeding stocks in this room, but did occur in a group of experimental animals which had been inoculated with material taken from an animal in this room. The presence of the virus was thus definitely established.

In this connection it is also of interest to note that before the presence of a contagious disease was recognized, several animals were transferred from this room to the breeding rooms; others were brought into the room for mating and then returned to their own quarters. None of these animals, however, developed pox until the epidemic was well advanced. Later, a litter of young 13 days old was taken from a

doe that had just died with pneumonia and transferred to a foster mother in A. A number of these animals died within 48 hours and they also showed pneumonia, but the doe which had fostered them and her own young were among the last in the colony to develop pox. The bearing of these transfers on the outbreak in the breeding rooms is, therefore, uncertain. It is of further interest in this connection that the mortality in D was not high and that the fulminating cases of pneumonia were largely limited to a group of Belgian hares and French silvers which were subsequently found to be among the most susceptible breeds.

The animals in Room C were not examined until after the first typical case of acute infection had been found in B. But, at this time, several cases were found. In addition, there were a number of animals with crusted and healing papular lesions of the skin and with lesions of the testicles in process of resolution. From these findings, it was evident that the disease had been present in mild form in this room for some time, thus establishing a second focus of infection antedating the outbreak in the two breeding rooms. It is possible, therefore, to trace the outbreak in the breeding rooms to either or both of these sources of infection.

The question then arises as to whether the infection in these rooms had an independent origin or spread from one to the other. This question cannot be answered with certainty. The evidence available indicates that there were two independent sources of infection which, in one case (Room D), gave rise to an acute fulminating type of infection and, in another (Room C), to a distinctly milder form of disease. The disease apparently developed at about the same time in both rooms and it was at least a week or 10 days before it spread to the breeding rooms.

The origin of the virus is also a matter of some uncertainty, but it is highly probable that in both cases infection originated from a passage virus. During the autumn, animals inoculated with neurovaccine and dermovaccine were kept on a lower floor of the building in which Room D is located and in rooms only a short distance down the corridor from the breeding colony. There was no report of a spontaneous spread of infection in any of these rooms until after the epidemic developed in the breeding colony, but it is known that in at

least one of them a disease presenting the clinical characteristics of rabbit pox was present earlier in the fall.

Isolation of inoculated animals is a difficult matter and ordinarily few or no precautions are taken to prevent the spread of the less harmful virus or bacterial infections among the animals of a given room or from one room to another. Caretakers provide a ready means for the dissemination of highly contagious material. In spite of this, serious outbreaks are comparatively rare. In the present instance, however, all available evidence indicated that from the passage of some virus, spontaneous spread did occur among the animals in one or more of these rooms. Eventually, the virus was transported in a highly virulent form to the nearest outposts of the breeding colony which was not protected in any way against outside infection. Spread of the infection continued from one room to another until practically the entire rabbit population of the Institute had been exposed, regardless of location or distance from the original source of infection and despite all efforts to quarantine or isolate groups of animals known to have been infected or exposed. The order of occurrence of outbreaks in various rooms and their relation to possible sources of infection are not known, since the recognition of the disease, in all cases, followed the discovery in the breeding colony.

Termination of the Epidemic

The course of events during the terminal stages of the epidemic brought out several points of epidemiological interest. As is usual during the early stages of an epidemic, the infection was highly contagious and the mortality correspondingly high, while, in the terminal stages, contagiousness diminished and the mortality was low. All of the adults and all of the young born prior to the epidemic except those that died of other causes developed pox. The same is true of all litters born during the early stages of the epidemic. There were, however, several instances of litters born after the epidemic was well under way in which no typical case of pox developed, and still others in which some animals developed typical cases of disease while other animals, despite the most intimate exposure, remained free from signs or symptoms of infection. In a third group of animals, known to have been exposed soon after birth, the incubation period was greatly

prolonged and lesions did not develop for several weeks; these infections were comparatively mild and were among the last cases of the epidemic. In most, if not all, of these exceptional cases the mother contracted the disease before or shortly after the birth of the litter and was not seriously affected. This sequence is apparently significant and suggests a passive protection from an immune mother. Still, the apparent change in contagiousness and definite decrease in the severity of the disease are difficult to explain.

The present tendency is to assume that conditions of this kind are brought about by a process of gradual immunization through exposure of the population to subinfecting doses of the etiological agent. In the present instance, however, there were comparatively few animals that escaped demonstrable infection, and the epidemic ended only after practically the entire population had contracted the disease. The few animals that apparently escaped infection were born after the epidemic began and were of an age found ordinarily to be highly susceptible. Some of them may have had extremely mild or asymptomatic infections or they may have become immune in the manner indicated above, which seems improbable. On the other hand, they may have been protected by nursing an immune mother, a passive rather than an active immunity.

At the time, it was not possible to carry out tests to determine whether the evident refractoriness of animals known to have been exposed was due to a specific immunity or to other causes. A year later, the immunity of some of these animals to vaccine virus was tested by Pearce, Rosahn and Hu as a part of an extensive series of experiments on the relation of vaccinia to rabbit pox. Vaccination showed that most of them were susceptible, but the significance of these findings is uncertain. The tests showed the condition of the animals at the end of a year and, at that time, their immunity was not of the same order as the immunity found in animals known to have recovered from rabbit pox. However, since the immunity produced by vaccination may be of short duration, it is also possible that these animals may have been protected by a transient immunity in the first instance, and the termination of the epidemic could still be explained by the development of a specific immunity in the entire population.

This explanation might appear to be the logical one if nothing more were known concerning the epidemiology of this disease. There was, however, a previous epidemic in 1930. In this instance, comparatively few animals contracted the disease and most of these recovered. At the time of the second epidemic, there were many animals in the colony that had passed through the first epidemic but none that were known to have had the disease. These animals proved to be just as susceptible to pox as the normal unexposed population so that, if they were protected in the first instance by a specific immunity, the immunity was again of short duration and afforded no appreciable protection against a second exposure.

Still a third epidemic of this disease occurred during the winter of 1933-34. This epidemic pursued a course like that of the first. It developed among experimental animals in a room containing a portion of the breeding colony, but its spread was limited and the mortality low. In this instance, young animals in the room were tested by Pearce, Rosahn and Hu immediately after the epidemic and found to be as susceptible as unexposed controls, so that the failure of the infection to spread could not be attributed to active immunization.

Finally, toward the end of the epidemic under consideration two experiments were carried out with young animals of the most susceptible age. These were obtained from outside sources and had not been exposed to rabbit pox. They were brought into the breeding rooms and exposed to infection by placing them in intimate contact with infected animals. All of them developed pox of a mild form but there were no deaths in a group of twelve animals. At the height of the epidemic the mortality among animals of this age group was upwards of 70 per cent. It is evident, therefore, that while the contagiousness was still high, the severity of the disease produced by natural infection was greatly diminished in animals which possessed no specific immunity.

These results, taken in conjunction with other evidence, cast considerable doubt on the significance of active and progressive immunization as the cause of the terminal abatement in the severity of the disease or as the sole cause for the termination of the epidemic. Old and young animals shared alike in the diminished severity of the disease which marked the terminal stages of the epidemic, and the evi-

dence available indicates that this characteristic change in the course of events was not due entirely, if at all, to a specific immunity but to some other group of factors which affected the entire population in a non-specific manner.

Epidemiological Considerations

The epidemic described is typical of epidemics in human populations and one of the chief points of interest is the opportunity afforded for a consideration of the rôle of epidemiological factors other than those concerned directly with the infectious agent. Accurate information of this kind is extremely difficult to obtain in human epidemics. It has been shown, however, that epidemic outbreaks in animal populations under experimental control are profoundly influenced by factors affecting the host as well as by factors which operate through the infecting organism (4). In experimental epidemics, efforts are directed toward the creation of known conditions and the maintenance of control so that the influence of given factors may be determined with accuracy. There is still some uncertainty, however, as to the conditions that actually prevail in spontaneous outbreaks and the relative importance of various epidemiological factors. Study of the present epidemic provides some information bearing on these points. The evidence collected is of two kinds. First, numerical data derived from systematic observations covering a long period of time and second, estimates of conditions based on judgment, both of which were originally intended to supply information concerning the health and activity of the community.

Reference to the data given above shows clearly that the reproductive activities of the adults in the population were not normal and that for a long time prior to the outbreak of rabbit pox the functional efficiency of these animals was on the decline. That this was not merely a disturbance of reproductive function is shown by parallel observations on the prevalence of disease.

The snuffles index is of particular interest in this connection. It concerns the same elements of the population and records the prevalence of a disease which is constantly present in the community. Here again, it was found that the severity and incidence of this disease began to increase well in advance of the pox epidemic with a

sharp rise immediately preceding the outbreak. There was, moreover, a definite spread of this disease to a portion of the population which is ordinarily insusceptible and this was associated with an increase in the death rate from pneumonia arising from infection with the same group of organisms.

Among the young animals, there was also evidence of low vitality indicated most clearly by the prevalence of gastro-intestinal disorders and deaths directly referable to these disorders. Here again, a disease which is ordinarily limited to certain elements of the population broke over the usual boundaries and caused the death of adults as well as young animals. The incidence of this disease also increased abruptly just previous to the epidemic invasion.

The significance of other abnormalities mentioned above need not be emphasized here. It is sufficient to say that the deterioration of the colony as evidenced by diminished functional efficiency and increased susceptibility to disease was unmistakable.

Experience has shown that for the most part conditions such as prevailed in the community prior to the epidemic are influenced by three groups of factors; namely, diet, cage accommodation or mode of living and climate or weather. Two of these are subject to control and their influence has been tested repeatedly.

The dietary aspect of these conditions is of particular interest. Some of the conditions do suggest a dietary deficiency. There is no reason, however, to assume that a diet found to be adequate for control or standard normal animals of the population was directly responsible for the sudden development of these abnormalities. It is true that the administration of vitamin concentrates usually affords relief in such a situation, but this has been interpreted as evidence of a variation in the vitamin requirement of the animals which results from fluctuations in unknown environmental factors.

It is important to note also that relief may be obtained by other means than diet. Adjustment of cage accommodations with improvement in the conditions of living has been found helpful in many cases. The conditions under consideration, therefore, cannot be regarded as dietary deficiencies in the usual sense. They are, rather, states of diminished vital capacity due to unknown causes, but susceptible of relief by improvement in the conditions of living in which diet plays a very important part.

It is of interest in this connection to note the variations in the disease picture which occurred in different epidemic stages. The first animals to contract the infection reacted with the production of a fulminating pneumonia and for some time the specific lesions of the disease were entirely visceral in distribution. During the height of the epidemic, lesions were widespread and cutaneous lesions formed the most typical clinical feature. Toward the end, however, visceral lesions were infrequent, there was a marked tendency to monosymptomatic affections and, in many cases, characteristic skin papules could be found only after a most extensive search. Similar variations in the disease picture are commonly found in human epidemics and suggest either a variation in the disease-producing properties of the infecting agent or a changing status on the part of the population.

In the present instance there was unmistakable evidence of an increased resistance in the animals during the terminal stages of the epidemic. This could hardly be explained on the basis of a specific immunity since the infection produced in animals of a highly susceptible age which had never been exposed to this infection was relatively harmless. It would have been desirable to have tested the status of the colony by the methods used in the pre-epidemic period. However, there was no means of gauging the functional efficiency of recovered animals until breeding operations were resumed. It was then found that fertility was surprisingly high, rose steadily to 100 per cent and for some time thereafter maintained an unprecendently high level. Among the earlier litters, however, there were still a considerable number of still born and non-viable young which were attributed to persistent abnormalities of the generative tract. But, on the whole, there was a striking improvement in reproductive efficiency and snuffles checks which were resumed about the same time showed a very low incidence of this disease.

It is impossible to prove or disprove a causal relation between the prevalence of a widespread and profound disturbance of the population and the occurrence of a devastating epidemic, or a relation between the terminal abatement in the severity of the disease and the coincident increase in resistance followed by general improvement in the condition of the population. But experiments carried out in these laboratories some years ago showed that the organic constitution of the rabbit is subject to a series of orderly variations which tend to

follow a seasonal course, modified by prevailing meteorological conditions (5). It was also found that susceptibility to experimentally induced disease varied with the prevailing condition of the animal organism and that both the organic constitution and the functional response to disease could be affected by modifying environmental conditions under experimental control. The question arises as to whether similar relations may not affect spontaneous outbreaks of disease.

Small pox is a winter disease and so also is rabbit pox. The evidence available indicates that rabbit pox is produced by a virus originating from vaccine virus. From the time the breeding colony was first organized (1929), it has been exposed to infection from inoculated animals in other laboratories through the same channels of communication which gave rise to the epidemic under consideration. So far, three epidemics of pox have occurred; two of them were mild and one was of devastating severity; two began during the 3rd week of December and the other in February. These facts indicate, on the one hand, a decided preference for the winter months and, on the other, a striking variation in the severity of disease on different occasions. Obviously, such peculiarities as these cannot be accounted for either on the basis of chance exposure to vaccine virus or by specific immunity. In view of the conditions which have existed over a period of years, it is even probable that exposure, comparable with that which gave rise to the epidemic reported, may have occurred repeatedly without producing a single typical case of disease. This is apparently in accord with the experience of those who have kept rabbits inoculated with vaccine virus in close proximity to animals that are not immune to this virus.

It is evident, therefore, that the chance introduction of vaccine virus into a non-immune population is not sufficient in itself to determine the occurrence of a severe epidemic; favorable conditions for the propagation of the infection are also essential. These probably include factors which favor an alteration in the pathogenic properties of the infecting organism, on the one hand, and diminished resistance of the host, on the other. Apparently, these two conditions are closely related and it is not improbable that both are referable to the operation of factors on the animal organism and they may be expressions of a common cause.

The experiments carried out by Pearce, Rosahn and Hu showed

that the virus isolated from the epidemic was related to vaccine virus but decidedly more virulent than the passage strain of neurovaccine with which it was compared and far more virulent than the dermo-vaccine. Under passage conditions this virulence was retained for a period of 9 months (January to September). During this time the infection in the colony died out. These results would indicate that under conditions of natural passage some alteration in the pathogenic properties of the pox virus had occurred. The characteristic properties of this virus are capable of perpetuation by parenteral inoculation, but when left to natural passage under conditions such as prevailed during the epidemic, reversion to a less virulent condition tends to occur and spread of the infection ceases.

One may conclude, therefore, that the decisive factors in the series of events associated with the development and termination of an epidemic are factors which affect the population. It has been shown experimentally that in the case of the rabbit, profound changes in organic constitution are induced by environmental factors and that susceptibility to disease varies accordingly. The series of events preceding the occurrence of the epidemic reported were expressions of a serious disturbance in the animal organism induced by some unknown environmental influence which is commonly operative at that period of the year but in this instance was of more than usual severity. The old idea of an "epidemic constitution" may find an explanation in the development of environmental conditions which affect the functional efficiency and disturb the health of a community and thus create conditions favorable to the spread of devastating infections. It is reasonably certain that factors of this kind played an important rôle in the epidemic of rabbit pox which has been reported.

SUMMARY

A devastating epidemic of rabbit pox in a breeding colony was studied with especial reference to factors of epidemiological significance.

The evidence obtained indicated that the epidemic originated among animals inoculated with vaccine virus and that the infection was spread to the breeding colony by caretakers. The epidemic began insidiously with atypical cases of visceral disease followed by typical

cases of pox and terminated as a mild cutaneous disease with scattering monosymptomatic affections of various kinds, difficult to recognize as cases of pox infection.

An analysis of data concerning the health and functional efficiency of the population and the immunity of exposed animals showed that the epidemic of rabbit pox was the terminal event in a series of progressive disorders which began fully a month before the first case of pox occurred. In like manner, the terminal decrease in the severity of the disease and the eventual termination of the epidemic appeared to be referable to an improvement in the condition of the population rather than to a specific immunity acquired by exposure to infection.

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MULTIPLICATION IN VITRO OF PSEUDORABIES VIRUS IN THE TESTICLE TISSUE OF IMMUNIZED GUINEA PIGS

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It is known that certain animal tissues, when removed from the animal, minced, and suspended in a suitable fluid medium, will remain alive for some time and can be experimentally infected with virus. With some viruses the infection can be recognized histologically by characteristic tissue changes; for example, the formation of inclusion bodies, hyperplasia, or necrosis. The pseudorabies virus does not cause such changes regularly, and the infection of the tissue can be definitely determined only by the results of inoculation of susceptible animals. In cultures of the virus with rabbit testicle tissue intranuclear inclusions were found in interstitial cells in the majority of the sections (1). In cultures with normal guinea pig testicle tissue intranuclear inclusions have been found in only one out of four series of cultures. They appeared in interstitial cells and were acidophilic like the inclusions in rabbit testicle cultures, but they were much fewer, smaller, and often fragmented. It was evident that in this instance the search for inclusion bodies could not replace the animal test for the presence of the virus in the cultures.

Tissue from immune animals can be tested for susceptibility to viruses only *in vitro*. So long as the tissue is in its physiological environment humoral, and possibly other immunity factors, interfere with the results.

Steinhart and Lambert (2) in 1914 used the tissue culture technique in the study of immunity. They found that vaccinia virus did not multiply in immune rabbit cornea tissue grown in immune plasma, but they did not determine whether the plasma or the tissue was responsible for the failure to grow. In 1929, Andrews (3, 4) and, simultaneously and independently, Rivers, Haagen, and Muckenfuss

(5) found that Virus III and vaccinia virus would form inclusion bodies in tissues *in vitro*. In Andrewes' experiments Virus III would readily grow and form inclusions in testicle tissue of immune rabbits in the presence of normal serum. In his later studies with herpes simplex (6), and the salivary gland virus of guinea pigs (7) similar results were obtained. With the salivary virus inclusions appeared in normal and immune tissue in the presence of normal serum, but the virus could not be subcultured. Rivers, Haagen, and Muckenfuss studied the formation of Guarneri bodies in cultures of rabbit cornea in rabbit plasma. In normal corneas soaked in virus for 3 hours and then transferred to immune plasma, Guarneri bodies developed. Immune corneas, however, even after washing, formed few or no inclusions when grown in normal plasma. Topacio and Hyde (8) in 1932 failed to confirm Andrewes' results with Virus III, although they used a similar technique. In their experiments immune rabbit testicle tissue in normal plasma could not be infected with virus even after washing with Tyrode solution. Immune plasma added to the cultures either before or after Virus III inhibited its growth in normal rabbit testis. The authors therefore tended to the conclusion that the immunity from Virus III infection was of both "the cellular and humoral types."

Because of the disagreement in the results obtained by different workers, an attempt was made to cultivate pseudorabies virus in tissue of immune guinea pigs. Testicle tissue was chosen because pseudorabies virus is known to multiply in this tissue from normal guinea pigs (1).

Methods

Immunization of Guinea Pigs

Immunization against pseudorabies is not a simple matter. Thus far it has failed with rabbits in this laboratory. Shope (personal communication) immunized guinea pigs against the Iowa (mad itch) strain of pseudorabies by repeated subcutaneous inoculations of sublethal doses of virus. He made the observation that this strain, when passed through a guinea pig (intracerebrally), became slightly attenuated for guinea pigs, and that subcutaneous inoculations of doses up to 800 mg. of the brain of such a guinea pig did not produce the disease in guinea pigs (9). When he gave guinea pigs four consecutive subcutaneous inoculations of 100 mg. infective guinea pig brain at 10 day intervals they resisted a subcutaneous inoculation of 100 mg. infective rabbit brain (approximately 100 M.L.D.) given 2 weeks after the last immunizing inoculation. Shope also found that, when an equally large dose of the more virulent Hungarian (Aujeszky) strain was used in the immunity test instead of the Iowa strain, not all of the treated guinea pigs would be immune.

Guinea pigs furnishing the testicle tissue used in the cultivation experiments were immunized according to Shope's method (see Table I). To increase the

degree of their immunity they were given additional inoculations of infective rabbit brain. The Iowa strain had been passed through a few more rabbits since Shope had performed his experiments and it had obviously increased in virulence for guinea pigs by such passages. Three guinea pigs died following the subcutaneous

TABLE I

Immunization of the Guinea Pigs, the Testes of Which Were Used in the Cultivation Experiment

Guinea pig No.	Subcutaneous immunizing inoculations										Immunity test			
	Iowa strain virus									Aujeszky strain		Intracerebral inoculation Apr. 1	Inoculation into right testis Mar. 26	
	Guinea pig brain					Rabbit brain				Rabbit brain				
	100 mg. Dec. 12, 1923	100 mg. Dec. 22	100 mg. Jan. 2, 1934	100 mg. Jan. 12	100 mg. Jan. 23	100 mg. Feb. 6	100 mg. Feb. 16	100 mg. Feb. 26	10 mg. Mar. 6	100 mg. Mar. 14	10 M.L.D.			100 M.L.D.
27-61	D													
26-77	D													
26-76	0	D												
28-66	0	Died of intercurrent disease												
27-63	0	0	0	0	0	0	0	0	0	0	D			
27-60	0	0	0	0	0	0	0	0	0	0	0	—	—	0
27-62	0	0	0	0	0	0	0	0	0	0	0	—	—	0
28-64	0	0	0	0	0	0	0	0	0	0	0	—	—	0
28-65	0	0	0	0	0	0	0	0	0	0	0	—	—	0
28-67	0	0	0	0	0	0	0	0	0	0	0	—	—	0
26-48	0	0	0	0	0	0	0	0	0	0	0	0	—	—
26-49	0	0	0	0	0	0	0	0	0	0	0	—	D	—
36-83														D
38-29														D
38-48												D		
36-19												D		
35-86													D	

D = died.

0 = No illness.

ous inoculation of a dose of infective guinea pig brain which previously was found to be non-fatal. Five guinea pigs were tested for immunity by the inoculation into the right testis of between 100 and 1000 M.L.D. of highly virulent pseudorabies virus cultivated in chicken embryo tissue (36th culture passage). All five guinea pigs resisted this inoculation. Two other guinea pigs were tested for immunity

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by intracerebral inoculation of Hungarian virus passed through the brain of a guinea pig. Guinea Pig 26-48 (Table I) resisted the inoculation of 10 M.L.D. (0.1 mg. virulent guinea pig brain), whereas Guinea Pig 26-49 succumbed to the inoculation of 100 M.L.D. (1 mg.) after a prolonged incubation period.

Preparation of Cultures

The testicle tissue of Guinea Pigs 28-64 and 28-65 used in the cultivation experiment was removed approximately 1 month after the intratesticular test for immunity. At that time the sera had strong neutralizing power, and the testicles did not contain virus demonstrable by the intracerebral inoculation of an emulsion of the carefully washed testicle tissue into mice. Control cultures were made with normal guinea pig testicle tissue.

The animals were killed and the testes were removed aseptically through the peritoneal cavity, finely minced with scissors in Petri dishes, and the tissue pulp was transferred with pipettes to large test tubes each containing 20 cc. Tyrode solution. After the tissue had been soaking in the solution for 10 minutes—during which time the tubes were occasionally shaken—the Tyrode solution was pipetted off, and replaced by an equal amount of fresh solution. This procedure was repeated twice. The tissue pulp was then transferred to 50 cc. Florence flasks (50 to 100 mg. per flask) containing mixtures of 3.2 cc. Tyrode solution + 0.8 cc. normal guinea pig serum. In a preliminary experiment, test tubes had been used as containers for the media, but the virus would not grow in them. The cultures of each group registered in Table II were made in triplicate.

Growth was initiated in each culture with 0.1 cc. of a Berkefeld V filtrate of ten ground cultures of pseudorabies virus in chicken embryo tissue (51st culture passage). The titer¹ of this filtrate was 1:1000. An amount of virus corresponding to approximately 100 M.L.D. for mice was thus added to each culture. According to a comparative titration experiment, this amount when inoculated intratesticularly into guinea pigs would correspond to about 1000 to 10,000 M.L.D. It would have been preferable not to inoculate more than 100 M.L.D. for guinea pigs (intratesticularly) into each culture, since the immunity of the guinea pigs used in this experiment was probably not an absolute one, and might have been overwhelmed by too large doses of virus (*vide* Guinea Pig 26-49, Table I). As will be seen from Table II, however, the titer of the first culture passage of group A, the most important one in the experiment, was low enough so that only an amount of virus corresponding roughly to from 4 to 40 M.L.D. (guinea pigs, intratesticularly) was transferred to the cultures of the second passage, and these became infected nevertheless as evidenced by the multiplication of the virus in them.

All cultures were incubated at 37.5°C. for 48 hours. The dilution factor between consecutive culture passages was 10. The titer of the cultures was de-

¹ By "titer" is meant the highest decimal dilution, 1 cc. of which killed mice when inoculated intraperitoneally.

terminated by intraperitoneal inoculations of undiluted ground cultures and decimal dilutions into white mice.

The titration results registered in Table II indicate that multiplication of the virus took place in all three groups of cultures. In normal guinea pig testis the virus always reached the highest concentration. In the culture with right immune testis the rate of multiplication was lower. In the group with left immune testis the virus was lost for an unknown reason after the fourth culture passage. Multiplication of the virus seems to have occurred in the cultures of this group, since there was no gradual decline of the titer from the first to the fourth passage but an equally high titer in the first, second, and third passages.

TABLE II

Cultivation of Pseudorabies Virus in Testicle Tissue from Immune Guinea Pigs

Culture passage No.	Group A		Group B		Group C	
	Cultures with right immune testis		Cultures with left immune testis		Cultures with normal testis	
	Tissue from guinea pig No.	Titer	Tissue from guinea pig No.	Titer	Tissue from guinea pig No.	Titer
1	28-64	1:1	28-64	1:10	40-73	At least 1:100
2	28-64	1:10	28-64	1:10	40-73	At least 1:1000
3	28-64	1:10	28-64	1:10	40-73	At least 1:1000
4	28-65	1:100	28-65	1:1	41-28	At least 1:1000
5	28-65	1:100	28-65	Avirulent	41-28	At least 1:1000
6	28-65	1:10	28-65	Avirulent	41-28	At least 1:1000

SUMMARY

Pseudorabies virus was cultivated *in vitro* in washed testicle tissue from immune guinea pigs, and evidence was thus procured which indicated that the testicle cells themselves had not become immune to pseudorabies. The rate of multiplication of the virus was considerably greater in control cultures with normal guinea pig testis than in cultures with immune testis. The reason for this fact may be that even by repeated washing the immune tissue could not be completely freed from fluid antibodies, and that such antibodies somewhat inhibited the multiplication of the virus.

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STUDIES ON THE SUPRARENAL CORTEX

IV. THE EFFECT OF SODIUM SALTS IN SUSTAINING THE SUPRARENALECTOMIZED DOG*

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A number of papers during the past few years, particularly those of Stewart and Rogoff (1), of Marine and Baumann (2), and of Loeb and his coworkers (3), have shown clearly the close relations which exist between the suprarenal glands and the metabolism of salt and water. The recent studies of Loeb are of fundamental interest since they provide for the first time a rational explanation of the mechanism of suprarenal insufficiency, which this author suggests may be due to a primary loss of sodium through the kidney.

The results which follow total ablation of the suprarenal glands in the dog have been confirmed by studies on the effects of withdrawal injections of the cortical hormone from the suprarenalectomized dog which had been previously maintained with healed wounds in a normal state of health and nutrition (4). Following the withdrawal of the hormone injections it has been shown that there is an increased urinary excretion of sodium, chloride, and of water, relative to the intake, which is presently reflected in a fall in the concentration of

We acknowledge the assistance of Dr. Oliver Kamm, of Parke, Davis and Co., who has generously supplied us for the past 4 years with the beef suprarenal glands which we prepare our cortical extract.

This investigation was aided in part by a grant from the Bingham Fund.

We wish to acknowledge the assistance of Dr. Mary Buell in supervising the technical procedures involved in the manufacture of the cortical extract and in the selection and setting up of analytical methods used in the studies herein reported. A preliminary report of portions of this work has been published (*Tr. Assn. Am. Phys.*, 1934, 49, 153).

these substances in the blood plasma. It would seem probable that an explanation of the prolonged survival which has been reported in suprarenalectomized hibernating animals (5) may be found in the lessened urinary excretion during the winter sleep, with a resulting diminished loss of sodium. It is possible that some additional mechanism for the conservation of sodium may be in part responsible for the prolonged survival also reported in suprarenalectomized animals during estrus or pregnancy (6).

The problem now arises as to whether this regulation of salt and water metabolism is the primary function of this cortical hormone obtained by extraction of beef suprarenal glands with lipoid solvents (7), or, on the other hand, whether there are other physiological derangements produced by removal of the suprarenal glands in the dog, which develop more slowly, and for this reason are usually masked by the earlier, more rapidly fatal effects of the dehydration associated with the loss of electrolytes.

The prolongation of the life of suprarenalectomized animals by injection of various fluids containing glucose or salts has been attempted by a number of workers, but the earlier experiments have never been entirely convincing.¹ The first demonstration of this effect of infusion was made by Brown-Séquard who showed in 1856 (8) that transfusion of blood from a normal into a suprarenalectomized animal will delay death for a considerable period. Marine and Baumann (2) in 1927 found that daily intraperitoneal injections of various sodium salts, for example normal sodium chloride solution, Ringer's solution, and isotonic sodium acetate, prolonged the lives of suprarenalectomized cats to about three times the span of untreated totally suprarenalectomized controls. They found further that sodium glycerophosphate was only slightly less effective. On the other hand, hypertonic saline solution, isotonic glucose solution, and glycerol had very little effect. They concluded that chloride is not an important factor even though a decrease in blood chlorides regularly occurred after suprarenalectomy. Although they pointed out the specific value of sodium salts in sustaining the animal they concluded that the action is only palliative, as all of the animals ultimately died of suprarenal insufficiency. The survival period did not exceed 15 days in any case.

Banting and Gairns (9), who observed a fall in blood urea after the injection of hypertonic saline (100 cc. 5 per cent NaCl), concluded that death could not be prevented even if blood urea, non-protein nitrogen, and chlorides are maintained at nearly normal levels.

¹ The earlier work is summarized by S. W. Britton (*Physiol. Rev.*, 1930, 10, 617).

Stewart and Rogoff (1), on the theory that suprarenal insufficiency is brought about by a severe and progressive intoxication, attempted the use of Ringer's solution, using 100 cc. per kilo per day, injected intravenously. Out of seventeen animals so treated, eight lived to the 18th day or longer, and of these three survived 30, 40, and 54 days respectively. Nausea, vomiting, and death eventually terminated all of their experiments. They concluded that "if it were practicable to wash out all of the poison it is conceivable that the animals would survive indefinitely," but that salt solution cannot "substitute" for the missing hormone.

In a recent paper Swingle and his collaborators (10) have studied the effect of feeding sodium chloride upon the life span of suprarenalectomized dogs. They found that intraperitoneal injections were ineffective in severe insufficiency, and so chose the oral route. Seven animals were used, but only three survived over 14 days. Of the three, one lived 19 days and one 21 days. The third animal was maintained for 50 days in excellent condition and only showed signs of insufficiency when the experiment was terminated, by cessation of the daily feeding of sodium chloride. Swingle concluded that it is not possible to sustain suprarenalectomized dogs without extract. The salt in his experiments was mixed with the food, and eventually the animals seem to have refused to eat the extremely salty rations.

The current view appears to be that salt and water are not effective substitutes for the cortical hormone (11). Some other factor seems to be regarded as essential, the nature of which is not clearly established.

The problem is important for an understanding of cortical function, and we have undertaken a series of experiments in which sodium has been supplied, both with and without added chloride, to the suprarenalectomized dog deprived of hormone injections. An attempt has been made to sustain so far as possible, the proper plasma level of the sodium ion. Under such circumstances, it was thought that the relatively uncontrolled renal excretion of sodium might be satisfied by an augmented exogenous supply, leaving the stores in the plasma and body fluids substantially intact, and hence, presumably, leaving undisturbed the normal conditions of tissue hydration. Granted the possibility that such proper electrolyte levels might be sustained by administration of added sodium and chloride ions alone, and with hormone injections completely withheld, we thought it possible that other physiological abnormalities due to cortical suprarenal deficiency, pointing to other essential functions of the suprarenal cortex or medulla might then be unmasked. Disorders of carbohydrate storage or of its mobilization might be disclosed, or nutritional deficiencies resulting

from the absence of the suprarenal cortical hormone, might be uncovered. It was thought possible that the increased plasma concentrations of potassium and of magnesium, which appear during suprarenal insufficiency, might progress unabated after withdrawal of hormone injections and at length prove toxic should they be phenomena occurring independent of the changes in sodium and chlorides.

On the other hand, the continued well-being of the suprarenalectomized animal when the level of plasma sodium is properly maintained without injections of the suprarenal cortical hormone would offer substantial evidence that regulation of electrolyte metabolism is its most essential function in the adult male dog.

It is necessary at the outset in such experiments to make sure that the amounts of sodium and chloride ions given to the animal are sufficient to repair the losses due to urinary excretion. This can best be ascertained by following the concentration of these electrolytes in the blood plasma at sufficiently frequent intervals. The results of our work indicate that suprarenalectomized adult male dogs may be sustained for prolonged periods without any cortical extract and without any apparent ill effect, provided that a balance be effected between intake and outgo of sodium and chloride ions so that no appreciable loss occurs at any time, and that no hemoconcentration takes place.

Following the withdrawal of injections of cortical hormone from the suprarenalectomized dog which is being given our usual diet of table scraps and occasional added beef stew and bones but without added salt, the excretion of urinary sodium exceeds that of chloride, and the fall in the plasma concentration (in milli-equivalents) is greater. From this observation it has seemed desirable that sodium should be supplied in excess of chloride to make up for this difference in the rate of excretion. That is, a mixture of sodium chloride plus sodium bicarbonate might be more effective in maintaining the normal plasma ion concentration in the suprarenalectomized animal over prolonged periods than sodium chloride alone. Such we have found to be the case. Indeed, adequate administration of sodium ion alone, as will be shown presently, can maintain proper blood concentration (ratio of plasma to cells) even though the plasma chloride drops to a low level. Eventually, however, such animals utterly refuse to eat and analysis

of the gastric juice after alcohol stimulation² shows a complete absence of free acid. It is possible that the low plasma chloride level may

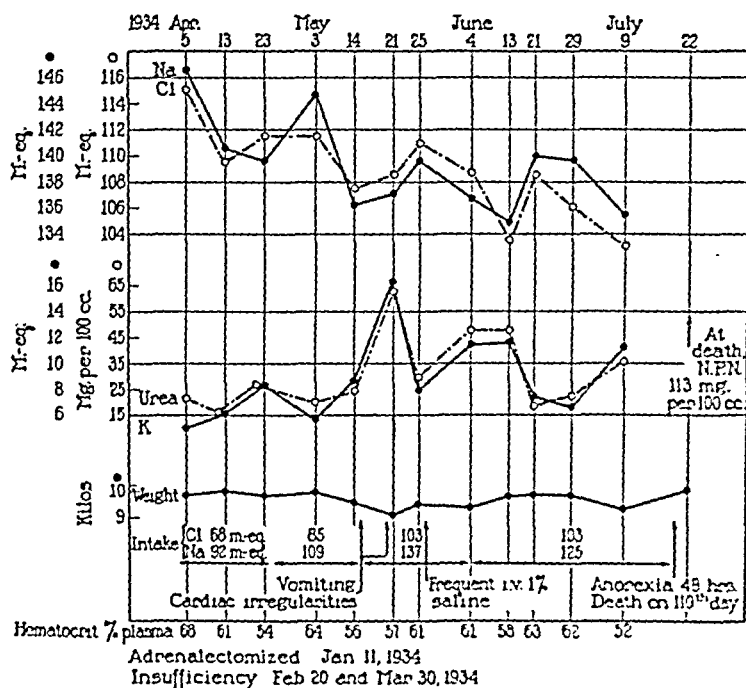


CHART 1. Dog 1-29. Adrenalectomized animal sustained 110 days (Apr. 5 to July 23) on sodium chloride and sodium bicarbonate only. Between May 14 and 25, vomiting and cardiac irregularities required additional intravenous saline. Thereafter the animal continued very well until July 9. The concentrations of sodium and chloride were low on July 9 (97th day) and hemoconcentration was indicated as well by the hematocrit reading. This indication for raising the intake of salt was unfortunately disregarded and the animal died 13 days later on July 22 after 72 hours of anorexia. Proper administration of sodium chloride and water would undoubtedly have prolonged this experiment indefinitely.

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² 50 cc. of 10 per cent ethyl alcohol are administered by stomach tube and the gastric contents are aspirated at the end of 15 and of 30 minutes. Practically no gastric juice may be obtained from the fasting animal without such stimulation. The use of histamine is not safe in suprarenalectomized dogs.

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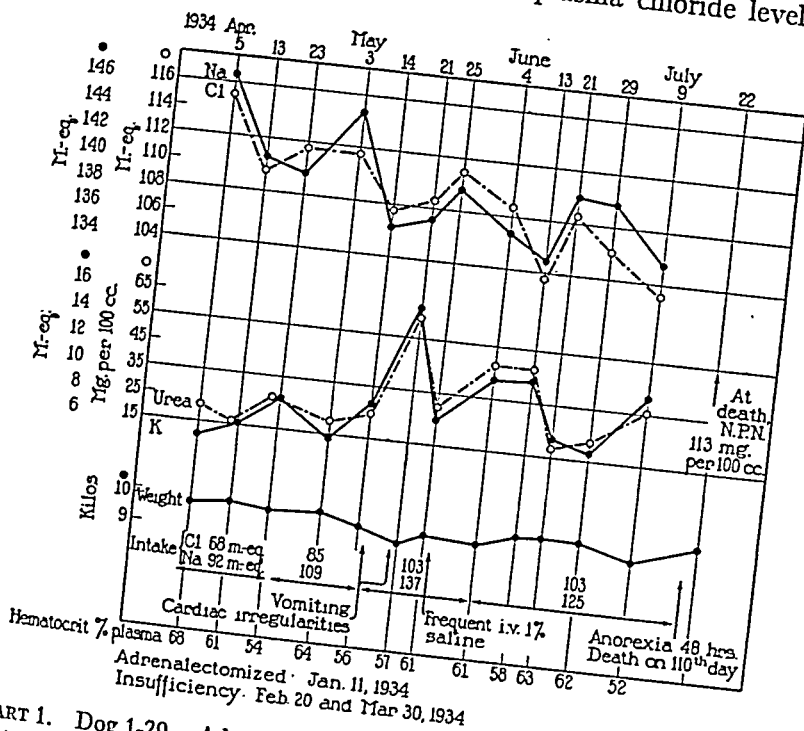


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maintenance of appetite. At any rate administration of sodium chloride in such animals immediately restores appetite coincidental

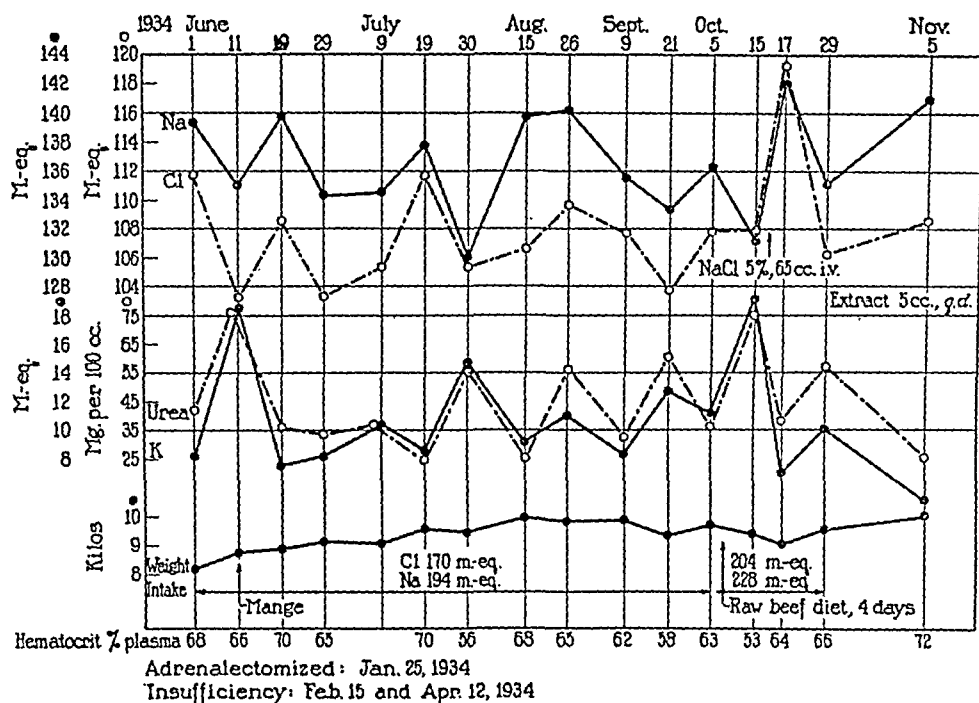


CHART 2. Dog 1-32. Adrenalectomized animal sustained for 152 days (June 1 to Oct. 29, 1934) on sodium chloride and sodium bicarbonate only. The diet was shifted on Oct. 9 to raw beef, resulting in anorexia, fall in plasma sodium and chloride, and rise in urea and potassium. The intravenous injection of 65 cc. 5 per cent sodium chloride on Oct. 15 restored the electrolyte pattern to approximately normal values with restoration of appetite and return of weight. The experiment was discontinued Oct. 29 on the 152nd day, when injections of cortical hormone were resumed. At this point the condition of the animal was excellent. At the end of 7 days (Nov. 5) the potassium had dropped and the sodium risen to normal values, with rise of 0.6 kilo in weight, indicating the more accurate electrolyte regulation possible only with injections of the cortical hormone. Both hormone and salt administration was then stopped, and the animal promptly went into insufficiency. Infection with mange, producing changes in pattern on June 11, was completely gone June 19.

with a rise in the plasma chloride level and the reappearance of free acid in the gastric juice.

Since appreciably greater amounts of sodium than of chlorides are lost, the result is that when sodium chloride (and hence equivalent

amounts of sodium and of chloride ions) alone is supplied in small quantities to the animal after the injections of the cortical hormone are stopped, a relatively greater loss of urinary sodium occurs producing a greater lowering of the plasma sodium level and hence a relative chloride acidosis. This acidosis may become uncompensated, and its occurrence may also have contributed to the failure of earlier attempts to maintain animals beyond a limited period by the administration of saline solutions alone.³

We report a group of experiments on suprarenalectomized dogs sustained by the oral administration of sodium and chloride ions for periods of 100 to 150 days (Charts 1 and 2, Table I). At the end of the period of experimentation, when properly conducted, the dogs do not differ in weight or activity from normal dogs or from suprarenalectomized dogs adequately sustained with injections of the cortical hormone. They appear to exhibit normal sexual activity, but the maintenance of normal fertility was not studied. Insufficiency has followed only when the salt administration was inadequate.⁴ A careful technique is required which has been developed after considerable experimentation.

Methods

Daily intravenous infusions, such as were made by Stewart and Rogoff (1), or intraperitoneal infusions, such as were employed by Marine and Baumann (2) over prolonged periods, were considered impractical for our purpose. In the latter case, the use of alkaline salts such as sodium bicarbonate intraperitoneally proved very irritating and sometimes fatal to our suprarenalectomized dogs. We turned, therefore, to the administration of the salts by mouth. If mixed with the diet, the large amounts that are frequently required resulted, as in Swingle's experience, in refusal to eat. Accordingly they were given by stomach tube dissolved in small amounts of water (5 per cent solution) in divided doses, twice a day, at 6 or 7 hour intervals, and never in association with the food. The animals were kept on the table for 20 to 30 minutes after gavage to prevent regurgitation.

³ Unpublished data supplied by Dr. K. Stuart Hetzel.

⁴ Postmortem study of animals sustained by sodium salts over prolonged periods without cortical hormone injections has shown no accessory cortical tissue present. No definite abnormalities were demonstrable in the pituitary, such as have been described by H. B. Schumacker and W. M. Firor (*Endocrinology*, 1934, 18, 676) in their adrenalectomized dog maintained with inadequate doses of the hormone.

TABLE I

Dog 1-22. Suprarenalectomy Jan. 11, 1934. Insufficiency Mar. 1, 1934, following withdrawal of extract injections. Prompt recovery with extract and salt.

Date	Day of experiment	Weight kg.	Non-protein nitrogen mg. per 100 cc.	Urea mg. per 100 cc.	Sugar mg. per 100 cc.	Plasma sodium per liter	Plasma potas- sium per liter	Plasma chlorides per liter	Plasma bicar- bonate per liter	Hema- to- crit plasma	Remarks
1934						mg.-eq.	mg.-eq.	mg.-eq.	m.-eq.	per 100 cc.	
Mar. 16		8.0	48	30	65	143.1	8.1	110.5	25.0	66.0	Injections of cortical hormone stopped Mar. 15. 5 gm. NaCl daily <i>per os</i>
26	10	8.3	52	28	52	138.8	8.7	107.9	20.3	62.5	5 gm. NaCl + 2 gm. NaHCO ₃ daily <i>per os</i>
Apr. 2	17	8.1	34	18	52	145.5	5.6	111.8	26.7	68.0	5 gm. NaCl + 2 gm. NaHCO ₃ daily <i>per os</i>
12	27	8.2	49	32	67	143.4	6.7	109.4	23.0	69.0	5 gm. NaCl + 2 gm. NaHCO ₃ daily <i>per os</i>
23	38	7.7	48	31	66	143.9	7.0	112.1	22.5	66.5	5 gm. NaCl + 2 gm. NaHCO ₃ daily <i>per os</i>
May 7	52	7.8	52	39	65	139.7	9.7	108.4	27.0	71.5	5 gm. NaCl + 2 gm. NaHCO ₃ daily <i>per os</i>
17	62	7.7	44	26	82	139.1	6.2	106.8	26.0	70.0	6 gm. NaCl + 2 gm. NaHCO ₃ daily <i>per os</i>
31	76	7.8	50	30	65	139.4	8.9	102.6	25.1	66.5	6 gm. NaCl + 2 gm. NaHCO ₃ daily <i>per os</i> Animal in excellent condition. Sustained from this point with NaHCO ₃ alone
June 8	84	7.9	39	22	60	140.3	6.3	99.9	37.0	67.5	No NaCl, 6 gm. NaHCO ₃ daily <i>per os</i> 100 cc. 5% NaHCO ₃ injected intravenously on this and on subsequent removal of blood samples for electrolyte study 6 gm. NaHCO ₃ daily <i>per os</i>

18	94	7.5	66	45	67	139.6	9.8	108.2	24.3	60.8	6 gm. NaHCO ₃ daily per os Some vomiting and diarrhea. Animal otherwise well NaHCO ₃ administration stopped Typical insufficiency, spasticity, low temperature
22	98	8.0	50	30	78					64.0	
25	101	7.7	100	69	45	134.9	13.7	104.0	25.6	60.0	
28	104	7.3	164	130	67	127.2	22.3	94.2	15.7	53.6	

Animal sustained 104 days (Mar. 16 to June 28, 1934) on sodium chloride and sodium bicarbonate only. From June 1 (77th day) the animal was sustained on sodium bicarbonate only, but the sodium chloride given in the food was evidently sufficient to maintain a proper plasma chloride concentration, except on June 8. From June 22 to 25 there was some vomiting and diarrhea. On June 25 (101st day) sodium bicarbonate administration stopped, anorexia appeared, and animal went into typical insufficiency during the following 72 hours. It was promptly restored to normal by the usual measures.

20	200	7.9									5	2	On May 19 appetite poor, and no food eaten May 20. Staggers. Pulse 40. Vomiting. Restored with extract and saline
21	20	8.0	112	75	80	134.2	17.3	106.3	17.8	60	7.0	5	2

The animal developed insufficiency on May 14 due to the administration of insufficient amounts of salt, the plasma sodium being sustained better than the chloride due to the larger ingestion of sodium. Infusion of 0.9 per cent sodium chloride lowered the potassium and urea concentration, reduced the hemoconcentration, and temporarily restored appetite. Eventually severe insufficiency occurred, however, because the salt ingestion by mouth was not adequate. In a subsequent experiment this animal was sustained with an adequate salt intake for 5 months without cortical extract (Chart 2).

Vomiting and diarrhea were avoided completely, and animals which did not retain the gavaged material readily were rejected. Failure in our preliminary studies was due primarily to the use of insufficient amounts of salt (Table II, Chart 3). The quantity required varies with the individual dog, and a proper dosage is necessary from the outset. This was regulated by the results of plasma electrolyte studies. The electrolyte pattern of the arterial blood plasma was determined at 7 to 10 day intervals, together with blood counts and hemoglobin, hematocrit, urea, and plasma protein estimations.⁵ Care was always taken to

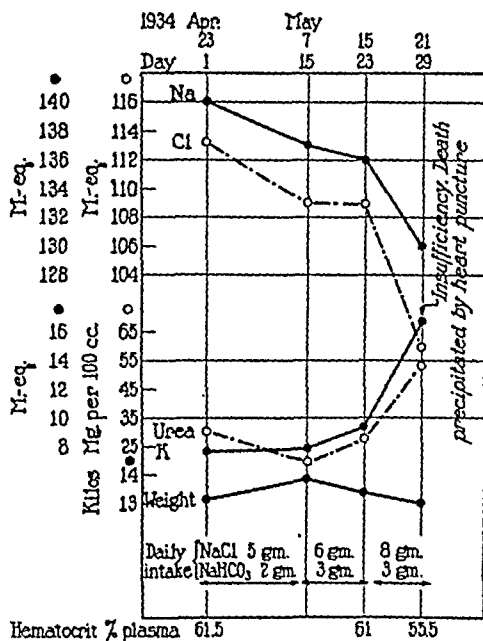


CHART 3. Dog 1-34. Suprarenalectomy Feb. 1, 1934. Typical changes in the electrolyte pattern, and the production of hemoconcentration resulting from an insufficient dosage of sodium salts in a suprarenalectomized dog receiving no extract.

replace the blood withdrawn by the intravenous injection of two or three times the quantity of 1 per cent salt solution. Occasionally further intravenous injection

⁵ The methods employed for the various chemical analyses have been previously described (4), with the following exception.

Plasma Potassium Method.—The method of S. E. Kerr (*J. Biol. Chem.*, 1926, 67, 689) was used for the deproteinization of serum and the estimation was made by the method of Kramer and Tisdall as described by J. P. Peters and D. D. Van Slyke (*Quantitative clinical chemistry. Volume II, Methods*, Baltimore, The Williams & Wilkins Co., 1932, 748).

tions of saline were given for a day or more when required, as indicated by the blood studies. Careful attention was paid to proper exercise, to proper food, to the avoidance of infections or parasites, and to cleanliness and proper shelter. The methods for the maintenance of our colony of suprarenalectomized dogs have been previously described (13).

TABLE III

Hypoglycemia without Insufficiency Produced by Fasting in a Suprarenalectomized Dog Receiving 4 Cc. Cortical Extract Daily and 0.5 Gm. Sodium Chloride

Dog 1-22. Suprarenalectomy Feb. 2, 1934. Repeatedly in insufficiency.

Date	Weight	Non-protein nitrogen	Urea	Plasma sodium per liter	Plasma potassium per liter	Plasma chlorides per liter	Plasma bicarbonate per liter	Plasma volume	Plasma proteins	Sugar	Remarks
1934	kg.	mg. per 100 cc.	mg. per 100 cc.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	per cent	mg. per 100 cc.	mg. per 100 cc.	
Aug. 23	7.7	32	18	137.9	5.9	112.1	24.5	71.0	5.4	65	No food after Aug. 22. Given 4 cc. cortical extract daily. 0.5 gm. NaCl per os
25	7.4	24	13							55	
27	7.1	22	13							45	
29	6.7	24	10							65	
31	6.5	22	10							62	
Sept. 2	6.3	28	14							57	
4	6.1	24	14							50	
5	6.0	24	14	143.0	5.7	113.0	22.7	70.9	5.6	35	Very weak. Experiment terminated. No convulsions. Alert on injection of intravenous glucose

The weight loss is greater in the animal receiving minimal amounts of salt, than in the animal sustained with salt alone (Table IV). There is no change in the percentage of plasma or in the plasma protein concentration. No evidence of dehydration or alteration of plasma electrolyte pattern when the experiment was terminated.

Mention has been made by Swingle (10) of the hypoglycemia observed in his experiments upon salt-fed dogs. This is often noted when the salt administered is inadequate in amount, and particularly when anorexia develops. Anorexia in animals treated with adequate amounts of cortical hormone may result in fatal hypoglycemia. The effect is clearly shown in fasted suprarenalectomized dogs sustained

either with cortical hormone together with minimal amounts of salt, or by means of adequate salt administration alone (Tables III, IV). In either case severe hypo-

TABLE IV

Hypoglycemia without Insufficiency Produced by Fasting in a Suprenalectomized Dog. Sustained with Salt Mixture but Given No Cortical Extract

Dog 1-41. Suprenalectomy May 4, 1934. Insufficiency June 1, 1934, and July 2, 1934.

Date	Weight	Non-protein nitrogen	Urea	Plasma sodium per liter	Plasma potassium per liter	Plasma chlorides per liter	Plasma bicarbonate per liter	Plasma volume	Plasma proteins	Sugar	Remarks
	kg.	mg. per 100 cc.	mg. per 100 cc.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	per cent	mg. per 100 cc.	mg. per 100 cc.	
1934											
July 30	8.8	46	25	143.8	8.4	112.2	18.8	70.5	6.5	62	No food after July 29. Given 6 gm. NaCl + 2 gm. NaHCO ₃ daily per os
Aug. 1	8.5	32	15							75	
3	8.6	25	11							68	
5	8.4	30	14							60	
7	8.0	30	17							68	
9 a.m.	7.8	30	17							38	Dog very dull. Pulse slow 35 to 40, irregular
9 p.m.		28	15	143.6	5.4	114.0		78.3	4.8	20	Experiment terminated. No convulsions. Lies in cage. Does not respond. Immediately after injection of 100 cc. 5% glucose animal became alert and active

As a result of fasting and salt feeding the percentage of plasma increased markedly and the plasma protein concentration dropped sharply. The plasma electrolyte pattern was not essentially altered.

glycemia occurs in 7 to 12 days, but without extracellular fluid loss. The plasma electrolyte pattern and the blood concentration are not altered. While either cortical hormone and salt, or salt alone, therefore, in proper amounts

will sustain the fasted animal and prevent hemoconcentration, neither will prevent the occurrence of severe hypoglycemia. The suprarenalectomized animal cannot form glycogen to maintain its blood sugar level from its own endogenous metabolism. When the glycogen supply is exhausted by fasting, hypoglycemia results. This, however, has not yet been established. It is a fact that the development of hypoglycemia is slower in the fasted, hormone-treated animal, but the differences, compared to the animal sustained with salt alone, are not very striking. This cortical hormone which influences mineral salt and water metabolism does not enable the dog to form carbohydrate from its own tissues. Hypoglycemia may be effectively prevented in animals sustained by salt alone by the daily use from the outset of certain lipoids, particularly cottonseed oil, or by the use of glucose, administered by stomach tube. Aside from the lack of food, an important cause of hypoglycemia in suprarenalectomized dogs is unusual physical exertion such as is brought about by fighting. Such an accident has caused sudden death in several of our animals, sustained without suprarenals over many months. As others have found, and as we have previously indicated, hypoglycemia is neither constant nor characteristic of suprarenal insufficiency in the dog, but is clearly related to the duration and extent of anorexia. The injection of glucose alone, or of glucose together with adrenalin, even though it raises the blood sugar level, does not relieve suprarenal insufficiency, nor influence the characteristic changes in the blood plasma in the male adult dog.

RESULTS

The maintenance of an approximately normal plasma concentration of sodium and chloride by the administration of adequate amounts of sodium and chloride ions is necessary for sustaining suprarenalectomized dogs without extract. This in turn sustains proper tissue hydration, that is to say, a sufficient supply of extracellular water, so that cell metabolism is unimpaired, the transfer of materials across cell membranes proceeds normally, and the intrinsic stores of intracellular water and dissolved substances are not interfered with. When this is effected there is no rise in plasma potassium nor urea concentration, no hemoconcentration, and, under proper conditions, no anorexia. When such proper tissue hydration is interfered with for a period, producing alteration in cellular permeability and a rise in plasma potassium and urea concentrations, adequate injections of sodium chloride may restore the normal plasma urea and potassium levels by facilitating urinary excretion of the excess. When the loss of sodium and the hemoconcentration is allowed to become extreme it is difficult and eventually impossible, without the use of the cortical hormone, to restore the animal to its normal state. When cell damage

TABLE V
High Plasma Sodium and Chloride Values in the Stage of Insufficiency Due to Concentration of the Plasma with Marked Loss of Weight
 Dog 1-32.

Degree of Insufficiency Due to Concentration of the Plasma with Marked Loss of Weight																
Date	Weight	Non-protein nitrogen		Urea		Plasma sodium per liter		Plasma potassium per liter		Plasma chloride per liter		Plasma bicarbonate per liter		Plasma volume	Remarks	
		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.			
1934																
Feb. 19	8.2	48	36	137.6	8.5	111.8	18	64	Injections of extract stopped Feb. 18. 4 gm. NaCl + 2 gm. NaHCO ₃ daily per os 4 gm. NaCl + 2 gm. NaHCO ₃ daily per os 5 gm. NaCl + 2 gm. NaHCO ₃ daily per os 5 gm. NaCl + 2 gm. NaHCO ₃ daily per os Loss of weight Classical symptoms with stagger in gait. Pulse 40, heart block. T. 95°F.							
26	8.1	47	36	133.0	8.7	106.8	20	62								
Mar. 6	8.1	75	56	133.4	18.5	102.7	21	60.5								
13	8.0	76	53	134.0	18.9	104.6	18.6	58.5								
17	7.3	76	50	150.1	18.3	129.2	17.0	60	Restored with extract and intravenous saline and glucose and salt by mouth. Pulse 40, heart block. T. 95°F.							
26	8.0	30	25	Restored with extract and intravenous saline and glucose and salt by mouth.				5.7								70
Apr. 4	8.0	48	25	141	9.0	112.4	18.5	61.5								
12	7.1	81	60	151.3	15.2	128.3	14.5	62								
13	6.95								Injections of extract stopped Mar. 25. 4 gm. NaCl + 2 gm. NaHCO ₃ + 20 mg. daily vitamin C intravenously T. 98°F. Stagger. Anorexia. Bradycardia Restored with extract, glucose and saline solution							
14	7.6	40	26	142.1	5.8	114.2	22.8	77								
Two cycles of insufficiency, the first of 27 days, weight (water) associated with anorexia, bradycardia, and marked loss of weight.																

Two cycles of insufficiency, the first of 27 days, the second of 16 days. In both cases marked hemoconcentration and loss of weight (water) associated with rise in concentration of sodium and chloride and rise in urea and potassium. Revival with extract, salt solution, and glucose produced lowering of sodium and chloride to normal levels. Treated with glucose and saline solution in prime condition.

has proceeded to an advanced stage a fatal outcome cannot be prevented by any treatment.

In a considerable proportion of animals which are fed salt in amounts insufficient to sustain proper tissue hydration, an actual terminal rise in the concentration of plasma sodium and of chloride occurs (Table V). Such animals have generally been maintained for several days on salt alone, the intake of fluids has been reduced, and urinary excretion is diminished. Hemoconcentration has taken place. The mechanism here involved may be analogous to that pointed out by Hartman (12), who observed that when urine excretion is too scanty, as in anhydremia due to diarrhea, the total electrolyte concentration in the plasma occasionally reaches enormous values. It may also be due to the administration of sodium chloride at too rapid a rate for the kidney of the suprarenalectomized animal to discharge with the water available for its excretion. In suprarenal insufficiency, coincidental with such increases in plasma concentration of sodium and of chloride, the urea and potassium values are also greatly increased. In each instance this rise in sodium and chloride concentrations was associated with a marked loss of body weight.

The question now arises as to the relative importance of the loss of sodium and of chloride in producing the dehydration and the associated symptoms, after withdrawal of the hormone injections. Experiments have been carried out in which the plasma sodium concentration was sustained by the use of sodium salts other than the chloride. For this purpose sodium bicarbonate in the quantity necessary proved unsatisfactory because it produced distention, vomiting, and diarrhea. Sodium gluconate and sodium lactate by mouth caused diarrhea. It was finally possible to use daily injections of suitable quantities of intravenous molar sodium N-lactate solution, prepared as directed by Hartman (14). Animals so sustained, without cortical hormone, maintained normal hydration, as indicated by hematocrit observations and protein studies (Table VI). A rapid fall in the plasma chlorides occurred, but the level was sustained when it reached about 90 m.-eq. per liter. No further fall occurred so long as the plasma sodium level was properly maintained. In these experiments an unsalted lean meat diet was used. Eventually anorexia appeared. The anorexia was associated with a diminution in the secretion of

Experiment with Intravenous $\text{m}/1$ Sodium Lactate. Sustained 18 Days until Anorexia Caused Marked Loss of Weight. Diet of Unsalted Raw Beef

TABLE VI

Dog 1-32.

Date	Weight kg.	Non-protein nitrogen		Urea		Plasma sodium per liter		Plasma potas- sium per liter		Plasma chlorides per liter		Plasma bicar- bonate per liter		Plasma proteins		Plasma volume		Remarks
		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	gm. per 100 cc.	gm. per 100 cc.	per 100 cc.				
1934																		
Nov. 15	9.7	60	40	136.6	7.3	105.8	25.4							6.4	66.1			Extract and sodium chloride stopped Nov. 14
19	9.4	112	100	135.0	9.0	99.8	26.2							7.1	62.0			50 cc. m/1 sodium lactate daily by vein + 3 gm. NaHCO ₃ per os
23	9.5	120	90	141.1	7.8	94.2	35.0							6.4	65.9			100 cc. m/1 sodium lactate daily by vein + 3 gm. NaHCO ₃ per os
27	9.5	108	82	137.8	8.4	98.0	28.0							6.5	68.0			
Dec. 3	9.0	44	30	138.1	4.9	93.0	34.5							6.0	63.6			150 cc. m/1 sodium lactate daily by vein + 3 gm. NaHCO ₃ per os. Anorexia
4	9.4	49	34	139.2	7.8	108.5	30.5							6.5	72			No free hydrochloric acid in gastric juice. Anorexia. Given 4 gm. NaCl per os. Blood sugar 75 mg. per 100 cc.
11	9.1	88	64	130.9	12.6	103.0	25.3							7.2	64.2			Free acid present in gastric juice. Ate 350 gm. beef 12 gm. NaCl + 2 gm. NaHCO ₃ per os. Diarrhea, Dec. 9-10

On Dec. 1 anorexia developed, but admini-
sodium level A

On Dec. 1 anorexia developed, but administration of increasing quantities of sodium lactate served to sustain the plasma sodium level. A lowered urea and potassium concentration resulted. The sodium concentration is sustained during the sodium lactate injections. On the evening of Dec. 3, no free acid was present in gastric contents. The dog was given 4 gm. sodium chloride by mouth, and free acid was present on Dec. 4, 14 hours later, coincidental with the rise to normal in plasma chloride concentration. The diarrhea on Dec. 9-10 was due to the cathartic action of the prolonged large doses of sodium with cortical extract and sodium chloride. The animal was restored

gastric juice and a disappearance of free hydrochloric acid. These studies afford further evidence that the sodium ion and not the chloride ion primarily governs tissue hydration in the suprarenalectomized dog, but that the concentration of plasma chloride has an intimate bearing upon the secretion of free hydrochloric acid in the stomach of the dog.

DISCUSSION

The experimental data which are presented indicate that an approximately normal concentration of both plasma sodium and chloride are required for the maintenance of the bilaterally suprarenalectomized dog. If the chloride falls anorexia appears and hypoglycemia eventually results. Fall in plasma sodium is accompanied by dehydration and hemoconcentration. Under the influence of the intact suprarenal, the sodium and chloride concentrations in the plasma (and of the extracellular fluid), are regulated with a high degree of constancy. It is not to be expected that in the absence of this regulatory mechanism it should be possible to supply additional sodium and chloride ions in amounts just sufficient to repair the body deficit resulting from their uncontrolled excretion, and thus to permit the salt and water metabolism to be maintained with the efficiency which it possesses in the normal presence of the natural hormone. Nevertheless the duration of the experiments which have been carried out should be sufficient to evoke other metabolic or nutritional disabilities resulting from the withdrawal of the cortical hormone and none has been demonstrated during experiments lasting from 3 to 5 months. Also it has been found in the treatment of Addison's disease by means of salt, that it is not possible in certain cases to maintain the normal plasma electrolyte levels by means of sodium salts alone, but that such a result is possible when injections of the hormone are given simultaneously (15). Similarly, it is only by the exhibition of both extract and salt in adequate amounts that entirely normal plasma electrolyte levels may be sustained in the totally suprarenalectomized dog.

Not only is the presence of the suprarenal cortex necessary for the maintenance of the constant normal concentration of plasma sodium in the dog when the intake is barely adequate or deficient, but the administration of excessive amounts of sodium, in the absence of sufficient

water available for its excretion, raises the plasma concentration of this ion appreciably above the normal level in the suprarenalectomized animal. The capacity of the normal kidney, depending upon the needs of the organism, to excrete sodium over a rather wide range is restricted with respect to the water simultaneously available for excretion. The level of this electrolyte in the plasma, and of the dissolved substances dependent upon it, in the animal sustained by salt alone, are, therefore, to a significant extent conditioned by the intake. Since the water available as a vehicle for the urinary excretion of dissolved substances, in the absence of the suprarenal cortical hormone, is particularly claimed by sodium and chloride, the excretion of other materials probably is impeded.

The fall in the concentrations of plasma sodium and chloride are usually accompanied, in these salt-treated animals, by a coincidental rise in the concentrations of plasma potassium and of urea, and conversely. These changes are usually parallel to each other (Charts 1, 2) and appear, as might be expected, to be at least approximately related to the extent of hemoconcentration. Infusion of sodium chloride solution, raising the plasma levels of sodium and of chloride, tends at the same time to restore the proper plasma concentrations of urea and potassium, by facilitating the urinary excretion of the excess. The effect of these reciprocal alterations in the plasma (extracellular fluid) concentrations must be to counteract or dampen the serious alterations in the osmotic pressure of the extracellular fluids attendant upon the disordered excretion of sodium and chloride. Such a buffering effect must make for stability of the fluid stores within extracellular and intracellular compartments.

We have elsewhere pointed out the probable relationship of the greatly increased potassium content of the plasma to the occurrence of cardiac arrhythmias during suprarenal insufficiency in the dog (16).

The possibility that various toxic effects due to potassium poisoning make their appearance during suprarenal insufficiency has been suggested by Hastings and Compere (17).

SUMMARY

1. A group of experiments is reported in which bilaterally suprarenalectomized adult male dogs have been maintained in apparently

normal condition over prolonged periods, up to 5 months, without the use of any suprarenal gland preparation or extract and by the administration of sodium chloride and sodium bicarbonate alone. Withdrawal of the salts then produced typical suprarenal insufficiency.

2. The relation of the absence of free hydrochloric acid in the gastric juice of suprarenalectomized animals, in addition to, or independent of the factor of dehydration, for the production of anorexia and hypoglycemia, is discussed.

3. Further evidence is presented in these experiments in support of the view that the suprarenal cortical hormone in the adult male dog is concerned with the regulation of sodium excretion by the kidney, and thus eventually with the proper maintenance of water balance in the organism. It has no direct influence on carbohydrate metabolism.

4. The reciprocal changes in the plasma concentrations of urea and of potassium which take place as the concentrations of plasma sodium and chlorides vary, are pointed out as furnishing a mechanism whereby abrupt alterations in osmotic pressure are dampened, and the volumes of fluids in extracellular and intracellular compartments more efficiently stabilized.

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